EXHIBIT 7

PART 2 OF 2

In one preferred embodiment, linker molecules are provided on a substrate. A terminal end of the linker molecules is provided with a reactive functional group protected with a photoremovable protective group. Using lithographic methods, the photoremovable protective group is exposed to light and removed from the linker molecules in first selected regions. The substrate is then washed or otherwise contacted with a first monomer that reacts with exposed functional groups on the linker molecules. In a preferred embodiment, the monomer is an amino acid containing a photoremovable protective group at its amino or carboxy terminus and the linker molecule terminates in an amino or carboxy acid group bearing a photoremovable protective group.

A second set of selected regions is, thereafter, exposed to light and the photoremovable protective group on the linker molecule/protected amino acid is removed at the second set of regions. The substrate is then contacted with a second monomer containing a photoremovable protective group for reaction with exposed functional groups. This process is repeated to selectively apply monomers until polymers of a desired length and desired chemical sequence are obtained. Photolabile groups are then optionally removed and the sequence is, thereafter, optionally capped. Side chain protective groups, if present, are also removed.

By using the lithographic techniques disclosed herein, it is possible to direct light to relatively small and precisely known locations on the substrate. It is, therefore, possible to synthesize polymers of a known chemical sequence at known locations on the substrate.

The resulting substrate will have a variety of uses including, for example, screening large numbers of polymers for biological activity. To screen for biological activity, the substrate is exposed to one or more receptors such as antibody whole cells, receptors on vesicles, lipids, or any one of a variety of other receptors. The receptors are preferably labeled with, for example, a fluorescent marker, radioactive marker, or a labeled antibody reactive with the receptor. The location of the marker on the substrate is detected with, for example, photon detection or autoradiographic techniques. Through knowledge of the sequence of the material at the location where binding is detected, it is possible to quickly determine which sequence binds with the receptor and, therefore, the technique can be used to screen large numbers of peptides. Other possible applications of the inventions herein include diagnostics in which various antibodies for particular receptors would be placed on a substrate and, for example, blood sera would be screened for immune deficiencies. Still further applications include, for example, selective "doping" of organic materials in semiconductor devices, and the like.

In connection with one aspect of the invention an improved reactor system for synthesizing polymers is also disclosed. The reactor system includes a substrate mount which engages a substrate around a periphery thereof. The substrate mount provides for a reactor space between the substrate and the mount through or into which reaction fluids are pumped or flowed. A mask is placed on or focused on the substrate and illuminated so as to deprotect selected regions of the substrate in the reactor space. A monomer is pumped through the reactor space or otherwise contacted with the substrate and reacts with the deprotected regions. By selectively deprotecting regions on the substrate and flowing predetermined monomers through the reactor space, desired polymers at known locations may be synthesized.

Improved detection apparatus and methods are also disclosed. The detection method and apparatus utilize a substrate having a large variety of polymer sequences at known locations on a surface thereof. The substrate is exposed to a fluorescently labeled receptor which binds to one or more of the polymer sequences. The substrate is placed in a microscope detection apparatus for identification of locations where binding takes place. The microscope detection apparatus includes a monochromatic or polychromatic light source for directing light at the substrate, means for detecting fluoreseed light from the substrate, and means for determining a location of the fluoresced light. The means for detecting light fluoresced on the substrate may in some embodiments include a photon counter. The means for determining a location of the fluoresced light may include an x/y translation table for the substrate. Translation of the slide and data collection are recorded and managed by an appropriately programmed digital computer.

A further understanding of the nature and advantages of the inventions herein may be realized by reference to the remaining portions of the specification and the attached drawings.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** illustrates masking and irradiation of a substrate at a first location. The substrate is shown in cross-section;

FIG. **2** illustrates the substrate after application of a monomer "A";

FIG. **3** illustrates irradiation of the substrate at a second location;

FIG. **4** illustrates the substrate after application of monomer "B";

FIG. **5** illustrates irradiation of the "All" monomer;

FIG. **6** illustrates the substrate after a second application of "B";

FIG. **7** illustrates a completed substrate;

FIGS. **8**A and **8**B illustrate alternative embodiments of a reactor system for forming a plurality of polymers on a substrate;

FIG. **9** illustrates a detection apparatus for locating fluorescent markers on the substrate;

FIGS. **10**A–**10**M illustrate the method as it is applied to the production of the trimers of monomers "A" and "B";

FIGS. **11**A and **11**B are fluorescence traces for standard fluorescent beads;

FIGS. **12**A and **12**B are fluorescence curves for NVOC slides not exposed and exposed to light respectively;

FIGS. **13**A to **13**D are fluorescence plots of slides exposed through 100 μm, 50 μm, 20 μm, and 10 μm masks;

FIG. **14** illustrates fluorescence of a slide with the peptide YGGFL on selected regions of its surface which has been exposed to labeled Herz antibody specific for this sequence;

FIGS. **15**A to **15**D illustrate formation of and a fluorescence plot of a slide with a checkerboard pattern of YGGFL and GGFL exposed to labeled Herz antibody. FIG. **15**C illustrates a 500×500 μm mask which has been focused on the substrate according to FIG. **8**A while FIG. **15**D illustrates a 50×50 μm mask placed in direct contact with the substrate in accord with FIG. **8**B;

FIG. **16** is a fluorescence plot of YGGFL and PGGFL synthesized in a 50 μm checkerboard pattern;

FIG. **17** is a fluorescence plot of YPGGFL and YGGFL synthesized in a 50 μm checkerboard pattern;

FIGS. **18**A and **18**B illustrate the mapping of sixteen sequences synthesized on two different glass slides;

FIG. **19** is a fluorescence plot of the slide illustrated in FIG. **18**A; and

FIG. **20** is a fluorescence plot of the slide illustrated in FIG. **10**B.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

CONTENTS

I. Glossary

The following terms are intended to have the following general meanings as they are used herein:

1. Complementary: Refers to the topological compatibility or matching together of interacting surfaces of a ligand molecule and its receptor. Thus, the receptor and its ligand can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.

2. Epitope: The portion of an antigen molecule which is delineated by the area of interaction with the subclass of receptors known as antibodies.

3. Ligand: A ligand is a molecule that is recognized by a particular receptor. Examples of ligands that can be investigated by this invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones (e.g., opiates, steroids, etc.), hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs, lectins, sugars, oligonucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.

4. Monomer: A member of the set of small molecules which can be joined together to form a polymer. The set of monomers includes but is not restricted to, for example, the set of common L-amino acids, the set of D-amino acids, the set of synthetic amino acids, the set of nucleotides and the set of pentoses and hexoses. As used herein, monomers refers to any member of a basis set for synthesis of a polymer. For example, dimers of L-amino acids form a basis set of 400 monomers for synthesis of polypeptides. Different basis sets of monomers may be used at successive steps in the synthesis of a polymer.

5. Peptide: A polymer in which the monomers are alpha amino acids and which are joined together through amide bonds and alternatively referred to as a polypeptide. In the context of this specification it should be appreciated that the amino acids may be the L-optical isomer or the D-optical isomer. Peptides are more than two amino acid monomers long, and often more than 20 amino acid monomers long. Standard abbreviations for amino acids are used (e.g., P for proline). These abbreviations are included in Stryer, *Biochemstry,* Third Ed., 1988, which is incorporated herein by reference for all purposes.

6. Radiation: Energy which may be selectively applied including energy having a wavelength of between 10^{-14} and 10^{4} meters including, for example, electron beam radiation, gamma radiation, x-ray radiation, ultra-violet radiation, visible light, infrared radiation, microwave radiation, and radio waves. "Irradiation" refers to the application of radiation to a surface.

7. Receptor: A molecule that has an affinity for a given ligand. Receptors may be naturally-occuring or manmade molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of receptors which can be employed by this invention include, but are not restricted to, antibodies, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells or other materials), drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles. Receptors are sometimes referred to in the art as anti-ligands. As the term receptors is used herein, no difference in meaning is intended. A "Ligand Receptor Pair" is formed when two macromolecules have combined through molecular recognition to form a complex.

Other examples of receptors which can be investigated by this invention include but are not restricted to:

a) Microorganism Receptors: Determination of ligands which bind to receptors, such as specific transport proteins or enzymes essential to survival of microorganisms, is useful in a new class of antibiotics. Of particular value would be antibiotics against opportunistic fungi, protozoa, and those bacteria resistant to the antibiotics in current use.

b) Enzymes: For instance, the binding site of enzymes such as the enzymes responsible for cleaving neurotransmitters; determination of ligands which bind to certain receptors to modulate the action of the enzymes which cleave the different neurotransmitters is useful in the development of drugs which can be used in the treatment of disorders of neurotransmission.

c) Antibodies: For instance, the invention may be useful in investigating the ligand-binding site on the antibody molecule which combines with the epitope of an antigen of interest; determining a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or

more of such sequences or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for auto-immune diseases (e.g., by blocking the binding of the "self" antibodies).

d) Nucleic Acids: Sequences of nucleic acids may be synthesized to establish DNA or RNA binding sequences.

e) Catalytic Polypeptides: Polymers, preferably polypeptides, which are capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products. Such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, which functionality is capable of chemically modifying the bound reactant. Catalytic polypeptides are described in, for example, U.S. application Ser. No. 404,920, which is incorporated herein by reference for all purposes.

f) Hormone receptors: For instance, the receptors for insulin and growth hormone. Determination of the ligands which bind with high affinity to a receptor is useful in the development of, for example, an oral replacement of the daily injections which diabetics must take to relieve the symptoms of diabetes, and in the other case, a replacement for the scarce human growth hormone which can only be obtained from cadavers or by recombinant DNA technology. Other examples are the vasoconstrictive hormone receptors; determination of those ligands which bind to a receptor may lead to the development of drugs to control blood pressure.

g) Opiate receptors: Determination of ligands which bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

8. Substrate: A material having a rigid or semi-rigid surface. In many embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, wells, raised regions, etched trenches, or the like. According to other embodiments, small beads may be provided on the surface which may be released upon completion of the synthesis.

9. Protective Group: A material which is bound to a monomer unit and which may be spatially removed upon selective exposure to an activator such as electromagnetic radiation. Examples of protective groups with utility herein include Nitroveratryloxy carbonyl, Nitrobenzyloxy carbonyl, Dimethyl dimethoxybenzyloxy carbonyl, 5-Bromo-7-nitroindolinyl, o-Hydroxy-α-methyl cinnamoyl, and 2-Oxymethylene anthraquinone. Other examples of activators include ion beams, electric fields, magnetic fields, electron beams, x-ray, and the like.

10. Predefined Region: A predefined region is a localized area on a surface which is, was, or is intended to be activated for formation of a polymer. The predefined region may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc. For the sake of brevity herein, "predefined regions" are sometimes referred to simply as "regions."

11. Substantially Pure: A polymer is considered to be "substantially pure" within a predefined region of a substrate when it exhibits characteristics that distinguish it from other predefined regions. Typically, purity will be measured in terms of biological activity or function as a result of uniform sequence. Such characteristics will typically be measured by way of binding with a selected ligand or receptor.

II. General

The present invention provides methods and apparatus for the preparation and use of a substrate having a plurality of polymer sequences in predefined regions. The invention is described herein primarily with regard to the preparation of molecules containing sequences of amino acids, but could readily be applied in the preparation of other polymers. Such polymers include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either α-, β-, or ω-amino acids, heteropolymers in which a known drug is covalently bound to any of the above, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this disclosure. In a preferred embodiment, the invention herein is used in the synthesis of peptides.

The prepared substrate may, for example, be used in screening a variety of polymers as ligands for binding with a receptor, although it will be apparent that the invention could be used for the synthesis of a receptor for binding with a ligand. The substrate disclosed herein will have a wide variety of other uses. Merely by way of example, the invention herein can be used in determining peptide and nucleic acid sequences which bind to proteins, finding sequence-specific binding drugs, identifying epitopes recognized by antibodies, and evaluation of a variety of drugs for clinical and diagnostic applications, as well as combinations of the above.

The invention preferably provides for the use of a substrate "S" with a surface. Linker molecules "L" are optionally provided on a surface of the substrate. The purpose of the linker molecules, in some embodiments, is to facilitate receptor recognition of the synthesized polymers.

Optionally, the linker molecules may be chemically protected for storage purposes. A chemical storage protective group such as t-BOC (t-butoxycarbonyl) may be used in some embodiments. Such chemical protective groups would be chemically removed upon exposure to, for example, acidic solution and would serve to protect the surface during storage and be removed prior to polymer preparation.

On the substrate or a distal end of the linker molecules, a functional group with a protective group P_0 is provided. The protective group P_0 may be removed upon exposure to radiation, electric fields, electric currents, or other activators to expose the functional group.

In a preferred embodiment, the radiation is ultraviolet (UV), infrared (IR), or visible light. As more fully described below, the protective group may alternatively be an electrochemically-sensitive group which may be removed in the presence of an electric field. In still further alternative embodiments, ion beams, electron beams, or the like may be used for deprotection.

In some embodiments, the exposed regions and, therefore, the area upon which each distinct polymer sequence is synthesized are smaller than about 1 cm^2 or less than 1 mm^2. In preferred embodiments the exposed area is less than about 10,000 μm^2 or, more preferably, less than 100 μm^2 and may, in some embodiments, encompass the binding site for as few as a single molecule. Within these regions, each polymer is preferably synthesized in a substantially pure form.

Concurrently or after exposure of a known region of the substrate to light, the surface is contacted with a first monomer unit M_1 which reacts with the functional group which has been exposed by the deprotection step. The first monomer includes a protective group P_1. P_1 may or may not be the same as P_0.

Accordingly, after a first cycle, known first regions of the surface may comprise the sequence:

$$S\text{-}L\text{-}M_1\text{-}P_1$$

while remaining regions of the surface comprise the sequence:

$$S\text{-}L\text{-}P_0.$$

Thereafter, second regions of the surface (which may include the first region) are exposed to light and contacted with a second monomer M_2 (which may or may not be the same as M_1) having a protective group P_2. P_2 may or may not be the same as P_0 and P_1. After this second cycle, different regions of the substrate may comprise one or more of the following sequences:

$$S—L—M_1—M_2—P_2 \qquad S—L—M_2—P_2$$

$$S—L—M_1—P_1 \quad \text{and/or} \quad S—L—P_0.$$

The above process is repeated until the substrate includes desired polymers of desired lengths. By controlling the locations of the substrate exposed to light and the reagents exposed to the substrate following exposure, the location of each sequence will be known.

Thereafter, the protective groups are removed from some or all of the substrate and the sequences are, optionally, capped with a capping unit C. The process results in a substrate having a surface with a plurality of polymers of the following general formula:

$$S\text{-}[L]\text{-}(M_i)\text{-}(M_j)\text{-}(M_k)\ldots(M_x)\text{-}[C]$$

where square brackets indicate optional groups, and $M_i \ldots M_x$ indicates any sequence of monomers. The number of monomers could cover a wide variety of values, but in a preferred embodiment they will range from 2 to 100.

In some embodiments a plurality of locations on the substrate polymers are to contain a common monomer subsequence. For example, it may be desired to synthesize a sequence $S\text{-}M_1\text{-}M_2\text{-}M_3$ at first locations and a sequence $S\text{-}M_4\text{-}M_2\text{-}M_3$ at second locations. The process would commence with irradiation of the first locations followed by contacting with M_1-P, resulting in the sequence $S\text{-}M_1$-P at the first location. The second locations would then be irradiated and contacted with M_4-P, resulting in the sequence $S\text{-}M_4$-P at the second locations. Thereafter both the first and second locations would be irradiated and contacted with the dimer $M_2\text{-}M_3$, resulting in the sequence $S\text{-}M_1\text{-}M_2\text{-}M_3$ at the first locations and $S\text{-}M_4\text{-}M_2\text{-}M_3$ at the second locations. Of course, common subsequences of any length could be utilized including those in a range of 2 or more monomers, 2 to 100 monomers, 2 to 20 monomers, and a most preferred range of 2 to 3 monomers.

According to other embodiments, a set of masks is used for the first monomer layer and, thereafter, varied light wavelengths are used for selective deprotection. For example, in the process discussed above, first regions are first exposed through a mask and reacted with a first monomer having a first protective group P_1, which is removable upon exposure to a first wavelength of light (e.g., IR). Second regions are masked and reacted with a second monomer having a second protective group P_2, which is removable upon exposure to a second wavelength of light (e.g., UV). Thereafter, masks become unnecessary in the synthesis because the entire substrate may be exposed alternatively to the first and second wavelengths of light in the deprotection cycle.

The polymers prepared on a substrate according to the above methods will have a variety of uses including, for example, screening for biological activity. In such screening activities, the substrate containing the sequences is exposed to an unlabeled or labeled receptor such as an antibody, receptor on a cell, phospholipid vesicle, or any one of a variety of other receptors. In one preferred embodiment the polymers are exposed to a first, unlabeled receptor of interest and, thereafter, exposed to a labeled receptor-specific recognition element, which is, for example, an antibody. This process will provide signal amplification in the detection stage.

The receptor molecules may bind with one or more polymers on the substrate. The presence of the labeled receptor and, therefore, the presence of a sequence which binds with the receptor is detected in a preferred embodiment through the use of autoradiography, detection of fluorescence with a charge-coupled device, fluorescence microscopy, or the like. The sequence of the polymer at the locations where the receptor binding is detected may be used to determine all or part of a sequence which is complementary to the receptor.

Use of the invention herein is illustrated primarily with reference to screening for biological activity. The invention will, however, find many other uses. For example, the invention may be used in information storage (e.g., on optical disks), production of molecular electronic devices, production of stationary phases in separation sciences, production of dyes and brightening agents, photography, and in immobilization of cells, proteins, lectins, nucleic acids, polysaccharides and the like in patterns on a surface via molecular recognition of specific polymer sequences. By synthesizing the same compound in adjacent, progressively differing concentrations, a gradient will be established to control chemotaxis or to develop diagnostic dipsticks which, for example, titrate an antibody against an increasing amount of antigen. By synthesizing several catalyst molecules in close proximity, more efficient multistep conversions may be achieved by "coordinate immobilization." Coordinate immobilization also may be used for electron transfer systems, as well as to provide both structural integrity and other desirable properties to materials such as lubrication, wetting, etc.

According to alternative embodiments, molecular biodistribution or pharmacokinetic properties may be examined. For example, to assess resistance to intestinal or serum proteases, polymers may be capped with a fluorescent tag and exposed to biological fluids of interest.

III. Polymer Synthesis

FIG. 1 illustrates one embodiment of the invention disclosed herein in which a substrate 2 is shown in cross-section. Essentially, any conceivable substrate may be employed in the invention. The substrate may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The substrate may have any convenient shape, such as a disc, square, sphere, circle, etc. The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which the synthesis takes place. The substrate and its surface preferably form a rigid support on which to carry out the reactions described herein. The substrate and its surface is also chosen to provide appropriate light-absorbing characteristics. For

instance, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO_2, SiN_4, modified silicon, or any one of a wide variety of gels or polymers such as (poly)tetrafluoroethylene, (poly) vinylidenedifluoride, polystyrene, polycarbonate, or combinations thereof. Other substrate materials will be readily apparent to those of skill in the art upon review of this disclosure. In a preferred embodiment the substrate is flat glass or single-crystal silicon with surface relief features of less than 10 Å.

According to some embodiments, the surface of the substrate is etched using well known techniques to provide for desired surface features. For example, by way of the formation of trenches, v-grooves, mesa structures, or the like, the synthesis regions may be more closely placed within the focus point of impinging light, be provided with reflective "mirror" structures for maximization of light collection from fluorescent sources, or the like.

Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials. In some embodiments the surface may provide for the use of caged binding members which are attached firmly to the surface of the substrate in accord with the teaching of copending application Ser. No. 404,920, previously incorporated herein by reference. Preferably, the surface will contain reactive groups, which could be carboxyl, amino, hydroxyl, or the like. Most preferably, the surface will be optically transparent and will have surface Si—OH functionalities, such as are found on silica surfaces.

The surface **4** of the substrate is preferably provided with a layer of linker molecules **6**, although it will be understood that the linker molecules are not required elements of the invention. The linker molecules are preferably of sufficient length to permit polymers in a completed substrate to interact freely with molecules exposed to the substrate. The linker molecules should be 6–50 atoms long to provide sufficient exposure. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing 2–10 monomer units, diamines, diacids, amino acids, or combinations thereof. Other-linker molecules may be used in light of this disclsoure.

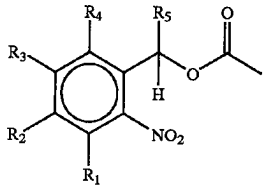
According to alternative embodiments, the linker molecules are selected based upon their hydrophilic/hydrophobic properties to improve presentation of synthesized polymers to certain receptors. For example, in the case of a hydrophilic receptor, hydrophilic linker molecules will be preferred so as to permit the receptor to more closely approach the synthesized polymer.

According to another alternative embodiment, linker molecules are also provided with a photocleavable group at an intermediate position. The photocleavable group is preferably cleavable at a wavelength different from the protective group. This enables removal of the various polymers following completion of the synthesis by way of exposure to the different wavelengths of light.

The linker molecules can be attached to the substrate via carbon—carbon bonds using, for example, (poly) trifluorochloroethylene surfaces, or preferably, by siloxane bonds (using, for example, glass or silicon oxide surfaces). Siloxane bonds with the surface of the substrate may be formed in one embodiment via reactions of linker molecules bearing trichlorosilyl groups. The linker molecules may optionally be attached in an ordered array, i.e., as parts of the head groups in a polymerized Langmuir Blodgett film. In alternative embodiments, the linker molecules are adsorbed to the surface of the substrate.

The linker molecules and monomers used herein are provided with a functional group to which is bound a protective group. Preferably, the protective group is on the distal or terminal end of the linker molecule opposite the substrate. The protective group may be either a negative protective group (i.e., the protective group renders the linker molecules less reactive with a monomer upon exposure) or a positive protective group (i.e., the protective group renders the linker molecules more reactive with a monomer upon exposure). In the case of negative protective groups an additional step of reactivation will be required. In some embodiments, this will be done by heating.

The protective group on the linker molecules may be selected from a wide variety of positive light-reactive groups preferably including nitro aromatic compounds such as o-nitrobenzyl derivatives or benzylsulfonyl. In a preferred embodiment, 6-nitroveratryloxycarbonyl (NVOC), 2-nitrobenzyloxycarbonyl (NBOC) or α,α-dimethyl-dimethoxybenzyloxycarbonyl (DDZ) is used. In one embodiment, a nitro aromatic compound containing a benzylic hydrogen ortho to the nitro group is used, i.e., a chemical of the form:



where R_1 is alkoxy, alkyl, halo, aryl, alkenyl, or hydrogen; R_2 is alkoxy, alkyl, halo, aryl, nitro, or hydrogen; R_3 is alkoxy, alkyl, halo, nitro, aryl, or hydrogen; R_4 is alkoxy, alkyl, hydrogen, aryl, halo, or nitro; and R_5 is alkyl, alkynyl, cyano, alkoxy, hydrogen, halo, aryl, or alkenyl. Other materials which may be used include o-hydroxy-α-methyl cinnamoyl derivatives. Photoremovable protective groups are described in, for example, Patchornik, *J. Am. Chem. Soc.* (1970) 92:6333 and Amit et al., *J. Org. Chem.* (1974) 39:192, both of which are incorporated herein by reference.

In an alternative embodiment the positive reactive group is activated for reaction with reagents in solution. For example, a 5-bromo-7-nitro indoline group, when bound to a carbonyl, undergoes reaction upon exposure to light at 420 nm.

In a second alternative embodiment, the reactive group on the linker molecule is selected from a wide variety of negative light-reactive groups including a cinammate group.

Alternatively, the reactive group is activated or deactivated by electron beam lithography, x-ray lithography, or any other radiation. Suitable reactive groups for electron beam lithography include sulfonyl. Other methods may be used including, for example, exposure to a current source. Other reactive groups and methods of activation may be used in light of this disclosure.

As shown in FIG. 1, the linking molecules are preferably exposed to, for example, light through a suitable mask 8 using photolithographic techniques of the type known in the semiconductor industry and described in, for example, Sze, *VLSI Technology,* McGraw-Hill (1983), and Mead et al., *Introduction to VLSI Systems,* Addison-Wesley (1980), which are incorporated herein by reference for all purposes. The light may be directed at either the surface containing the

protective groups or at the back of the substrate, so long as the substrate is transparent to the wavelength of light needed for removal of the protective groups. In the embodiment shown in FIG. **1**, light is directed at the surface of the substrate containing the protective groups. FIG. **1** illustrates the use of such masking techniques as they are applied to a positive reactive group so as to activate linking molecules and expose functional groups in areas **10***a* and **10***b*.

The mask **8** is in one embodiment a transparent support material selectively coated with a layer of opaque material. Portions of the opaque material are removed, leaving opaque material in the precise pattern desired on the substrate surface. The mask is brought into close proximity with, imaged on, or brought directly into contact with the substrate surface as shown in FIG. **1**. "openings" in the mask correspond to locations on the substrate where it is desired to remove photoremovable protective groups from the substrate. Alignment may be performed using conventional alignment techniques in which alignment marks (not shown) are used to accurately overlay successive masks with previous patterning steps, or more sophisticated techniques may be used. For example, interferometric techniques such as the one described in Flanders et al., "A New Interferometric Alignment Technique," *App. Phys. Lett.* (1977) 31:426–428, which is incorporated herein by reference, may be used.

To enhance contrast of light applied to the substrate, it is desirable to provide contrast enhancement materials between the mask and the substrate according to some embodiments. This contrast enhancement layer may comprise a molecule which is decomposed by light such as quinone diazid or a material which is transiently bleached at the wavelength of interest. Transient bleaching of materials will allow greater penetration where light is applied, thereby enhancing contrast. Alternatively, contrast enhancement may be provided by way of a cladded fiber optic bundle.

The light may be from a conventional incandescent source, a laser, a laser diode, or the like. If non-collimated sources of light are used it may be desirable to provide a thick- or multi-layered mask to prevent spreading of the light onto the substrate. It may, further, be desirable in some embodiments to utilize groups which are sensitive to different wavelengths to control synthesis. For example, by using groups which are sensitive to different wavelengths, it is possible to select branch positions in the synthesis of a polymer or eliminate certain masking steps. Several reactive groups along with their corresponding wavelengths for deprotection are provided in Table 1.

TABLE 1

Group	Approximate Deprotection Wavelength
Nitroveratryloxy carbonyl (NVOC)	UV (300–400 nm)
Nitrobenzyloxy carbonyl (NBOC)	UV (300–350 nm)
Dimethyl dimethoxybenzyloxy carbonyl	UV (280–300 nm)
5-Bromo-7-nitroindolinyl	UV (420 nm)
o-Hydroxy-α-methyl cinnamoyl	UV (300–350 nm)
2-Oxymethylene anthraquinone	UV (350 nm)

While the invention is illustrated primarily herein by way of the use of a mask to illuminate selected regions the substrate, other techniques may also be used. For example, the substrate may be translated under a modulated laser or diode light source. Such techniques are discussed in, for example, U.S. Pat. No. 4,719,615 (Feyrer et al.), which is incorporated herein by reference. In alternative embodiments a laser galvanometric scanner is utilized. In other embodiments, the synthesis may take place on or in contact with a conventional liquid crystal (referred to herein as a "light valve") or fiber optic light sources. By appropriately modulating liquid crystals, light may be selectively controlled so as to permit light to contact selected regions of the substrate. Alternatively, synthesis may take place on the end of a series of optical fibers to which light is selectively applied. Other means of controlling the location of light exposure will be apparent to those of skill in the art.

The substrate may be irradiated either in contact or not in contact with a solution (not shown) and is, preferably, irradiated in contact with a solution. The solution contains reagents to prevent the by-products formed by irradiation from interfering with synthesis of the polymer according to some embodiments. Such by-products might include, for example, carbon dioxide, nitrosocarbonyl compounds, styrene derivatives, indole derivatives, and products of their photochemical reactions. Alternatively, the solution may contain reagents used to match the index of refraction of the substrate. Reagents added to the solution may further include, for example, acidic or basic buffers, thiols, substituted hydrazines and hydroxylamines, reducing agents (e.g., NADH) or reagents known to react with a given functional group (e.g., aryl nitroso+glyoxylic acid→aryl formhydroxamate+CO_2).

Either concurrently with or after the irradiation step, the linker molecules are washed or otherwise contacted with a first monomer, illustrated by "A" in regions **12***a* and **12***b* in FIG. **2**. The first monomer reacts with the activated functional groups of the linkage molecules which have been exposed to light. The first monomer, which is preferably an amino acid, is also provided with a photoprotective group. The photoprotective group on the monomer may be the same as or different than the protective group used in the linkage molecules, and may be selected from any of the above-described protective groups. In one embodiment, the protective groups for the A monomer is selected from the group NBOC and NVOC.

As shown in FIG. **3**, the process of irradiating is thereafter repeated, with a mask repositioned so as to remove linkage protective groups and expose functional groups in regions **14***a* and **14***b* which are illustrated as being regions which were protected in the previous masking step. As an alternative to repositioning of the first mask, in many embodiments a second mask will be utilized. In other alternative embodiments, some steps may provide for illuminating a common region in successive steps. As shown in FIG. **3**, it may be desirable to provide separation between irradiated regions. For example, separation of about 1–5 μm may be appropriate to account for alignment tolerances.

As shown in FIG. **4**, the substrate is then exposed to a second protected monomer "B," producing B regions **16***a* and **16***b*. Thereafter, the substrate is again masked so as to remove the protective groups and expose reactive groups on A region **12***a* and B region **16***b*. The substrate is again exposed to monomer B, resulting in the formation of the structure shown in FIG. **6**. The dimers B-A and B-B have been produced on the substrate.

A subsequent series of masking and contacting steps similar to those described above with A (not shown) provides the structure shown in FIG. **7**. The process provides all possible dimers of B and A, i.e., B-A, A-B, A-A, and B-B.

The substrate, the area of synthesis, and the area for synthesis of each individual polymer could be of any size or shape. For example, squares, ellipsoids, rectangles, triangles, circles, or portions thereof, along with irregular geometric-shapes, may be utilized. Duplicate synthesis areas may also be applied to a single substrate for purposes of redundancy.

In one embodiment the regions **12** and **16** on the substrate will have a surface area of between about 1 cm^2 and 10^{-10} cm^2. In some embodiments the regions **12** and **16** have areas of less than about 10^{-1} cm^2, 10^{-2} cm^2, 10^{-3} cm^2, 10^{-4} cm^2, 10^{-5} cm^2, 10^{-6} cm^2, 10^{-7} cm^2, 10^{-8} cm^2, or 10^{-10} cm^2. In a preferred embodiment, the regions **12** and **16** are between about 10×10 μm and 500×500 μm.

In some embodiments a single substrate supports more than about 10 different monomer sequences and perferably more than about 100 different monomer sequences, although in some embodiments more than about 10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 different sequences are provided on a substrate. Of course, within a region of the substrate in which a monomer sequence is synthesized, it is preferred that the monomer sequence be substantially pure. In some embodiments, regions of the substrate contain polymer sequences which are at least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% pure.

According to some embodiments, several sequences are intentionally provided within a single region so as to provide an initial screening for biological activity, after which materials within regions exhibiting significant binding are further evaluated.

IV. Details of One Embodiment of a Reactor System

FIG. **8**A schematically illustrates a preferred embodiment of a reactor system **100** for synthesizing polymers on the prepared substrate in accordance with one aspect of the invention. The reactor system includes a body **102** with a cavity **104** on a surface thereof. In preferred embodiments the cavity **104** is between about 50 and 1000 μm deep with a depth of about 500 μm preferred.

The bottom of the cavity is preferably provided with an array of ridges **106** which extend both into the plane of the Figure and parallel to the plane of the Figure. The ridges are preferably about 50 to 200 μm deep and spaced at about 2 to 3 mm. The purpose of the ridges is to generate turbulent flow for better mixing. The bottom surface of the cavity is preferably light absorbing so as to prevent reflection of impinging light.

A substrate **112** is mounted above the cavity **104**. The substrate is provided along its bottom surface **114** with a photoremovable protective group such as NVOC with or without an intervening linker molecule. The substrate is preferably transparent to a wide spectrum of light, but in some embodiments is transparent only at a wavelength at which the protective group may be removed (such as UV in the case of NVOC). The substrate in some embodiments is a conventional microscope glass slide or cover slip. The substrate is preferably as thin as possible, while still providing adequate physical support. Preferably, the substrate is less than about 1 mm thick, more preferably less than 0.5 mm thick, more preferably less than 0.1 mm thick, and most preferably less than 0.05 mm thick. In alternative preferred embodiments, the substrate is quartz or silicon.

The substrate and the body serve to seal the cavity except for an inlet port **108** and an outlet port **110**. The body and the substrate may be mated for sealing in some embodiments with one or more gaskets. According to a preferred embodiment, the body is provided with two concentric gaskets and the intervening space is held at vacuum to ensure mating of the substrate to the gaskets.

Fluid is pumped through the inlet port into the cavity by way of a pump **116** which may be, for example, a model no. B-120-S made by Eldex Laboratories. Selected fluids are circulated into the cavity by the pump, through the cavity, and out the outlet for recirculation or disposal. The reactor may be subjected to ultrasonic radiation and/or heated to aid in agitation in some embodiments.

Above the substrate **112**, a lens **120** is provided which may be, for example, a 2" 100 mm focal length fused silica lens. For the sake of a compact system, a reflective mirror **122** may be provided for directing light from a light source **124** onto the substrate. Light source **124** may be, for example, a Xe(Hg) light source manufactured by Oriel and having model no. 66024. A second lens **126** may be provided for the purpose of projecting a mask image onto the substrate in combination with lens **112**. This form of lithography is referred to herein as projection printing. As will be apparent from this disclosure, proximity printing and the like may also be used according to some embodiments.

Light from the light source is permitted to reach only selected locations on the substrate as a result of mask **128**. Mask **128** may be, for example, a glass slide having etched chrome thereon. The mask **128** in one embodiment is provided with a grid of transparent locations and opaque locations. Such masks may be manufactured by, for example, Photo Sciences, Inc. Light passes freely through the transparent regions of the mask, but is reflected from or absorbed by other regions. Therefore, only selected regions of the substrate are exposed to light.

As discussed above, light valves (LCD's) may be used as an alternative to conventional masks to selectively expose regions of the substrate. Alternatively, fiber optic faceplates such as those available from Schott Glass, Inc, may be used for the purpose of contrast enhancement of the mask or as the sole means of restricting the region to which light is applied. Such faceplates would be placed directly above or on the substrate in the reactor shown in FIG. **8**A. In still further embodiments, flys-eye lenses, tapered fiber optic faceplates, or the like, may be used for contrast enhancement.

In order to provide for illumination of regions smaller than a wavelength of light, more elaborate techniques may be utilized. For example, according to one preferred embodiment, light is directed at the substrate by way of molecular microcrystals on the tip of, for example, micropipettes. Such devices are disclosed in Lieberman et al., "A Light Source Smaller Than the Optical Wavelength," *Science* (1990) 247:59–61, which is incorporated herein by reference for all purposes.

In operation, the substrate is placed on the cavity and sealed thereto. All operations in the process of preparing the substrate are carried out in a room lit primarily or entirely by light of a wavelength outside of the light range at which the protective group is removed. For example, in the case of NVOC, the room should be lit with a conventional dark room light which provides little or no UV light. All operations are preferably conducted at about room temperature.

A first, deprotection fluid (without a monomer) is circulated through the cavity. The solution preferably is of 5 mM sulfuric acid in dioxane solution which serves to keep exposed amino groups protonated and decreases their reactivity with photolysis by-products. Absorptive materials such as N,N-diethylamino 2,4-dinitrobenzene, for example, may be included in the deprotection fluid which serves to absorb light and prevent reflection and unwanted photolysis.

The slide is, thereafter, positioned in a light raypath from the mask such that first locations on the substrate are illuminated and, therefore, deprotected. In preferred embodiments the substrate is illuminated for between about 1 and 15 minutes with a preferred illumination time of about 10 minutes at 10–20 mW/cm^2 with 365 nm light. The slides are neutralized (i.e., brought to a pH of about 7) after

photolysis with, for example, a solution of di-isopropylethylamine (DIEA) in methylene chloride for about 5 minutes.

The first monomer is then placed at the first locations on the substrate. After irradiation, the slide is removed, treated in bulk, and then reinstalled in the flow cell. Alternatively, a fluid containing the first monomer, preferably also protected by a protective group, is circulated through the cavity by way of pump **116**. If, for example, it is desired to attach the amino acid Y to the substrate at the first locations, the amino acid Y (bearing a protective group on its α-nitrogen), along with reagents used to render the monomer reactive, and/or a carrier, is circulated from a storage container **118**, through the pump, through the cavity, and back to the inlet of the pump.

The monomer carrier solution is, in a preferred embodiment, formed by mixing of a first solution (referred to herein as solution "A") and a second solution (referred to herein as solution "B"). Table 2 provides an illustration of a mixture which may be used for solution A.

TABLE 2

Representative Monomer Carrier Solution "A"	
100 mg	NVOC amino protected amino acid
37 mg	HOBT (1-Hydroxybenzotriazole)
250 μl	DMF (Dimethylformamide)
86 μl	DIEA (Diisopropylethylamine)

The composition of solution B is illustrated in Table 3. Solutions A and B are mixed and allowed to react at room temperature for about 8 minutes, then diluted with 2 ml of DMF, and 500 μl are applied to the surface of the slide or the solution is circulated through the reactor system and allowed to react for about 2 hours at room temperature. The slide is then washed with DMF, methylene chloride and ethanol.

TABLE 3

Representative Monomer Carrier Solution "B"	
250 μl	DMF
111 mg	BOP (Benzotriazolyl-n-oxy-tris (dimethylamino) phosphoniumhexafluorophosphate)

As the solution containing the monomer to be attached is circulated through the cavity, the amino acid or other monomer will react at its carboxy terminus with amino groups on the regions of the substrate which have been deprotected. Of course, while the invention is illustrated by way of circulation of the monomer through the cavity, the invention could be practiced by way of removing the slide from the reactor and submersing it in an appropriate monomer solution.

After addition of the first monomer, the solution containing the first amino acid is then purged from the system. After circulation of a sufficient amount of the DMF/methylene chloride such that removal of the amino acid can be assured (e.g., about 50× times the volume of the cavity and carrier lines), the mask or substrate is repositioned, or a new mask is utilized such that second regions on the substrate will be exposed to light and the light **124** is engaged for a second exposure. This will deprotect second regions on the substrate and the process is repeated until the desired polymer sequences have been synthesized.

The entire derivatized substrate is then exposed to a receptor of interest, preferably labeled with, for example, a fluorescent marker, by circulation of a solution or suspension of the receptor through the cavity or by contacting the surface of the slide in bulk. The receptor will preferentially bind to certain regions of the substrate which contain complementary sequences.

Antibodies are typically suspended in what is commonly referred to as "supercocktail," which may be, for example, a solution of about 1% BSA (bovine serum albumin), 0.5% Tween in PBS (phosphate buffered saline) buffer. The antibodies are diluted into the supercocktail buffer to a final concentration of, for example, about 0.1 to 4 μg/ml.

FIG. **8**B illustrates an alternative preferred embodiment of the reactor shown in FIG. **8**A. According to this embodiment, the mask **128** is placed directly in contact with the substrate. Preferably, the etched portion of the mask is placed face down so as to reduce the effects of light dispersion. According to this embodiment, the imaging lenses **120** and **126** are not necessary because the mask is brought into close proximity with the substrate.

For purposes of increasing the signal-to-noise ratio of the technique, some embodiments of the invention provide for exposure of the substrate to a first labeled or unlabeled receptor followed by exposure of a labeled, second receptor (e.g., an antibody) which binds at multiple sites on the first receptor. If, for example, the first receptor is an antibody derived from a first species of an animal, the second receptor is an antibody derived from a second species directed to epitopes associated with the first species. In the case of a mouse antibody, for example, fluorescently labeled goat antibody or antiserum which is antimouse may be used to bind at multiple sites on the mouse antibody, providing several times the fluorescence compared to the attachment of a single mouse antibody at each binding site. This process may be repeated again with additional antibodies (e.g., goat-mouse-goat, etc.) for further signal amplification.

In preferred embodiments an ordered sequence of masks is utilized. In some embodiments it is possible to use as few as a single mask to synthesize all of the possible polymers of a given monomer set.

If, for example, it is desired to synthesize all 16 dinucleotides from four bases, a 1 cm square synthesis region is divided conceptually into 16 boxes, each 0.25 cm wide. Denote the four monomer units by A, B, C, and D. The first reactions are carried out in four vertical columns, each 0.25 cm wide. The first mask exposes the left-most column of boxes, where A is coupled. The second mask exposes the next column, where B is coupled; followed by a third mask, for the C column; and a final mask that exposes the right-most column, for D. The first, second, third, and fourth masks may be a single mask translated to different locations.

The process is repeated in the horizontal direction for the second unit of the dimer. This time, the masks allow exposure of horizontal rows, again 0.25 cm wide. A, B, C, and D are sequentially coupled using masks that expose horizontal fourths of the reaction area. The resulting substrate contains all 16 dinucleotides of four bases.

The eight masks used to synthesize the dinucleotide are related to one another by translation or rotation. In fact, one mask can be used in all eight steps if it is suitably rotated and translated. For example, in the example above, a mask with a single transparent region could be sequentially used to expose each of the vertical columns, translated 90°, and then sequentially used to allow exposure of the horizontal rows.

Tables 4 and 5 provide a simple computer program in Quick Basic for planning a masking program and a sample output, respectively, for the synthesis of a polymer chain of three monomers ("residues") having three different monomers in the first level, four different monomers in the second level, and five different monomers in the third level in a

striped pattern. The output of the program is the number of cells, the number of "stripes" (light regions) on each mask, and the amount of translation required for each exposure of the mask.

TABLE 4

Mask Strategy Program

```
DEFINT A-Z
DIM b(20), w(20), 1(500)
F$ = "LPT1:"
OPEN f$ FOR OUTPUT AS #1
jmax = 3 'Number of residues
b(1) = 3: b(2) = 4: b(3) = 5 'Number of building blocks for res 1,2,3
g = 1: 1max(1) = 1
FOR j = 1 TO jmax: g= g * b(j): NEXT j
w(0) = 0: w(1) = g / b(l)
PRINT #1, "MASK2.BAS ", DATE$, TIME$: PRINT #1,
PRINT #1, USING "Number of residues=##"; jmax
FOR j = 1 TO jmax
PRINT #1, USING "      Residue ##      ##building blocks"; j; b(j)
NEXT j
PRINT #1, "
PRINT #1, USING "Number of cells=####"; g: PRINT #1,
FOR j = 2 TO jmax
1max(j) = lmax(j - 1) * b(j - 1)
w(j) = w(j - 1) / b(j)
NEXT j
FOR j = 1 TO jmax
PRINT #1, USING "Mask for residue ##"; j: PRINT #1,
PRINT #1, USING "    Number of stripes =###"; 1max(j)
PRINT #1, USING "    Width of each stripe =###"; w(j)
FOR 1 = 1 TO lmax(j)
a = 1 + (1 - 1) * w(j - 1)
ae = a + w(j) - 1
PRINT #1, USING " Stripe ## begins at
location ### and ends at ###"; 1; a; ae
NEXT 1
PRINT #1,
PRINT #1, USING " For each of ## building blocks, translate mask by ##
cell(s)"; b(j); w(j),
PRINT #1, : PRINT #1, : PRINT #1,
NEXT j
```


TABLE 5

Masking Strategy Output

```
Number of residues= 3
Residue 1                          3 building blocks
Residue 2                          4 building blocks
Residue 3                          5 building blocks
Number of cells= 60
Mask for residue 1

Number of stripes= 1
Width of each stripe= 20
Stripe 1 begins at location    1 and ends at 20
For each of 3 building blocks, translate mask by 20 cell(s)
Mask for residue 2

Number of stripes= 3
Width of each stripe= 5
Stripe 1 begins at location        1 and ends at 5
Stripe 2 begins at location       21 and ends at 25
Stripe 3 begins at location       41 and ends at 45
For each of 4 building blocks, translate mask by 5 cell(s)
Mask for residue 3

Number of stripes= 12
Width of each stripe= 1
Stripe  1 begins at location       1 and ends at  1
Stripe  2 begins at location       6 and ends at  6
Stripe  3 begins at location      11 and ends at 11
Stripe  4 begins at location      16 and ends at 16
Stripe  5 begins at location      21 and ends at 21
```

TABLE 5-continued

Masking Strategy Output

```
Stripe  6 begins at location      26 and ends at 26
Stripe  7 begins at location      31 and ends at 31
Stripe  8 begins at location      36 and ends at 36
Stripe  9 begins at location      41 and ends at 41
Stripe 10 begins at location      46 and ends at 46
Stripe 11 begins at location      51 and ends at 51
Stripe 12 begins at location      56 and ends at 56
For each of 5 building blocks, translate mask by 1 cell(s)
```


V. Details of One Embodiment of A Fluorescent Detection Device

FIG. **9** illustrates a fluorescent detection device for detecting fluorescently labeled receptors on a substrate. A substrate **112** is placed on an x/y translation table **202**. In a preferred embodiment the x/y translation table is a model no. PM500-A1 manufactured by Newport Corporation. The x/y translation table is connected to and controlled by an appropriately programmed digital computer **204** which may be, for example, an appropriately programmed IBM PC/AT or AT compatible computer. Of course, other computer systems, special purpose hardware, or the like could readily be substituted for the AT computer used herein for illustration. Computer software for the translation and data collection functions described herein can be provided based on commercially available software including, for example, "Lab Windows" licensed by National Instruments, which is incorporated herein by reference for all purposes.

The substrate and x/y translation table are placed under a microscope **206** which includes one or more objectives **208**. Light (about 488 nm) from a laser **210**, which in some embodiments is a model no. 2020-05 argon ion laser manufactured by Spectraphysics, is directed at the substrate by a dichroic mirror **207** which passes greater than about 520 nm light but reflects 488 nm light. Dichroic mirror **207** may be, for example, a model no. FT510 manufactured by Carl Zeiss. Light reflected from the mirror then enters the microscope **206** which may be, for example, a model no. Axioscop 20 manufactured by Carl Zeiss. Fluorescein-marked materials on the substrate will fluoresce >488 nm light, and the fluoresced light will be collected by the microscope and passed through the mirror. The fluorescent light from the substrate is then directed through a wavelength filter **209** and, thereafter through an aperture plate **211**. Wavelength filter **209** may be, for example, a model no. OG530 manufactured by Melles Griot and aperture plate **211** may be, for example, a model no. 477352/477380 manufactured by Carl Zeiss.

The fluoresced light then enters a photomultiplier tube **212** which in some embodiments is a model no. R943-02 manufactured by Hamamatsu, the signal is amplified in preamplifier 214 and photons are counted by photon counter **216**. The number of photons is recorded as a function of the location in the computer **204**. Pre-Amp **214** may be, for example, a model no. SR440 manufactured by Stanford Research Systems and photon counter **216** may be a model no. SR400 manufactured by Stanford Research Systems. The substrate is then moved to a subsequent location and the process is repeated. In preferred embodiments the data are acquired every 1 to 100 μm with a data collection diameter of about 0.8 to 10 μm preferred. In embodiments with sufficiently high fluorescence, a CCD detector with broad-field illumination is utilized.

By counting the number of photons generated in a given area in response to the laser, it is possible to determine where

fluorescent marked molecules are located on the substrate. Consequently, for a slide which has a matrix of polypeptides, for example, synthesized on the surface thereof, it is possible to determine which of the polypeptides is complementary to a fluorescently marked receptor.

According to preferred embodiments, the intensity and duration of the light applied to the substrate is controlled by varying the laser power and scan stage rate for improved signal-to-noise ratio by maximizing fluorescence emission and minimizing background noise.

While the detection apparatus has been illustrated primarily herein with regard to the detection of marked receptors, the invention will find application in other areas. For example, the detection apparatus disclosed herein could be used in the fields of catalysis, DNA or protein gel scanning, and the like.

VI. Determination of Relative Binding Strength of Receptors

The signal-to-noise ratio of the present invention is sufficiently high that not only can the presence or absence of a receptor on a ligand be detected, but also the relative binding affinity of receptors to a variety of sequences can be determined.

In practice it is found that a receptor will bind to several peptide sequences in an array, but will bind much more strongly to some sequences than others. Strong binding affinity will be evidenced herein by a strong fluorescent or radiographic signal since many receptor molecules will bind in a region of a strongly bound ligand. Conversely, a weak binding affinity will be evidenced by a weak fluorescent or radiographic signal due to the relatively small number of receptor molecules which bind in a particular region of a substrate having a ligand with a weak binding affinity for the receptor. Consequently, it becomes possible to determine relative binding avidity (or affinity in the case of univalent interactions) of a ligand herein by way of the intensity of a fluorescent or radiographic signal in a region containing that ligand.

Semiquantitative data on affinities might also be obtained by varying washing conditions and concentrations of the receptor. This would be done by comparison to known ligand receptor pairs, for example.

VII. Examples

The following examples are provided to illustrate the efficacy of the inventions herein. All operations were conducted at about ambient temperatures and pressures unless indicated to the contrary.

A. Slide Preparation

Before attachment of reactive groups it is preferred to clean the substrate which is, in a preferred embodiment a glass substrate such as a microscope slide or cover slip. According to one embodiment the slide is soaked in an alkaline bath consisting of, for example, 1 liter of 95% ethanol with 120 ml of water and 120 grams of sodium hydroxide for 12 hours. The slides are then washed under running water and allowed to air dry, and rinsed once with a solution of 95% ethanol.

The slides are then aminated with, for example, aminopropyltriethoxysilane for the purpose of attaching amino groups to the glass surface on linker molecules, although any omega functionalized silane could also be used for this purpose. In one embodiment 0.1% aminopropyltriethoxysilane is utilized, although solutions with concentrations from 10^{-7}% to 10% may be used, with about 10^{-3}% to 2% preferred. A 0.1% mixture is prepared by adding to 100 ml of a 95% ethanol/5% water mixture, 100 microliters (μl) of aminopropyltriethoxysilane. The mixture is agitated at about ambient temperature on a rotary shaker for about 5 minutes. 500 μl of this mixture is then applied to the surface of one side of each cleaned slide. After 4 minutes, the slides are decanted of this solution and rinsed three times by dipping in, for example, 100% ethanol.

After the plates dry, they are placed in a 110–120° C. vacuum oven for about 20 minutes, and then allowed to cure at room temperature for about 12 hours in an argon environment. The slides are then dipped into DMF (dimethylformamide) solution, followed by a thorough washing with methylene chloride.

The aminated surface of the slide is then exposed to about 500 μl of, for example, a 30 millimolar (mM) solution of NVOC-GABA (gamma amino butyric acid) NHS (N-hydroxysuccinimide) in DMF for attachment of a NVOC-GABA to each of the amino groups.

The surface is washed with, for example, DMF, methylene chloride, and ethanol.

Any unreacted aminopropyl silane on the surface—that is, those amino groups which have not had the NVOC-GABA attached—are now capped with acetyl groups (to prevent further reaction) by exposure to a 1:3 mixture of acetic anhydride in pyridine for 1 hour. Other materials which may perform this residual capping function include trifluoroacetic anhydride, formicacetic anhydride, or other reactive acylating agents. Finally, the slides are washed again with DMF, methylene chloride, and ethanol.

B. Synthesis of Eight Trimers of "A" and "B"

FIG. **10** illustrates a possible synthesis of the eight trimers of the two-monomer set: gly, phe (represented by "A" and "B," respectively). A glass slide bearing silane groups terminating in 6-nitro-veratryloxycarboxamide (NVOC-NH) residues is prepared as a substrate. Active esters (pentafluorophenyl, OBt, etc.) of gly and phe protected at the amino group with NVOC are prepared as reagents. While not pertinent to this example, if side chain protecting groups are required for the monomer set, these must not be photoreactive at the wavelength of light used to protect the primary chain.

For a monomer set of size n, n×ρ cycles are required to synthesize all possible sequences of length ρ. A cycle consists of:

1. Irradiation through an appropriate mask to expose the amino groups at the sites where the next residue is to be added, with appropriate washes to remove the by-products of the deprotection.
2. Addition of a single activated and protected (with the same photochemically-removable group) monomer, which will react only at the sites addressed in step 1, with appropriate washes to remove the excess reagent from the surface.

The above cycle is repeated for each member of the monomer set until each location on the surface has been extended by one residue in one embodiment. In other embodiments, several residues are sequentially added at one location before moving on to the next location. Cycle times will generally be limited by the coupling reaction rate, now as short as 20 min in automated peptide synthesizers. This step is optionally followed by addition of a protecting group to stabilize the array for later testing. For some types of polymers (e.g., peptides), a final deprotection of the entire surface (removal of photoprotective side chain groups) may be required.

More particularly, as shown in FIG. **10**A, the glass **20** is provided with regions **22**, **24**, **26**, **28**, **30**, **32**, **34**, and **36**. Regions **30**, **32**, **34**, and **36** are masked, as shown in FIG. **10**B and the glass is irradiated and exposed to a reagent

containg "A" (e.g., gly), with the resulting structure shown in FIG. **10**C. Thereafter, regions **22**, **24**, **26**, and **28** are masked, the glass is irradiated (as shown in FIG. **10**D) and exposed to a reagent containing "B" (e.g., phe), with the resulting structure shown in FIG. **10**E. The process proceeds, consecutively masking and exposing the sections as shown until the structure shown in FIG. **10**M is obtained. The glass is irradiated and the terminal groups are, optionally, capped by acetylation. As shown, all possible trimers of gly/phe are obtained.

In this example, no side chain protective group removal is necessary. If it is desired, side chain deprotection may be accomplished by treatment with ethanedithiol and trifluoroacetic acid.

In general, the number of steps needed to obtain a particular polymer chain is defined by:

$$n \times e \quad (1)$$

where:

n=the number of monomers in the basis set of monomers, and

e=the number of monomer units in a polymer chain.

Conversely, the synthesized number of sequences of length e will be:

$$n^e. \quad (2)$$

Of course, greater diversity is obtained by using masking strategies which will also include the synthesis of polymers having a length of less than e. If, in the extreme case, all polymers having a length less than or equal to e are synthesized, the number of polymers synthesized will be:

$$n^e+n^{e-1}+\ldots+n^1. \quad (3)$$

The maximum number of lithographic steps needed will generally be n for each "layer" of monomers, i.e., the total number of masks (and, therefore, the number of lithographic steps) needed will be nxe. The size of the transparent mask regions will vary in accordance with the area of the substrate available for synthesis and the number of sequences to be formed. In general, the size of the synthesis areas will be:

$$\text{size of synthesis areas}=(A)/(S)$$

where:

A is the total area available for synthesis; and

S is the number of sequences desired in the area.

It will be appreciated by those of skill in the art that the above method could readily be used to simultaneously produce thousands or millions of oligomers on a substrate using the photolithographic techniques disclosed herein. Consequently, the method results in the ability to practically test large numbers of, for example, di, tri, tetra, penta, hexa, hepta, octapeptides, dodecapeptides, or larger polypeptides (or correspondingly, polynucleotides).

The above example has illustrated the method by way of a manual example. It will of course be appreciated that automated or semi-automated methods could be used. The substrate would be mounted in a flow cell for automated addition and removal of reagents, to minimize the volume of reagents needed, and to more carefully control reaction conditions. Successive masks could be applied manually or automatically.

C. Synthesis of a Dimer of an Aminopropyl Group and a Fluorescent Group

In synthesizing the dimer of an aminopropyl group and a fluorescent group, a functionalized durapore membrane was used as a substrate. The durapore membrane was a polyvinylidine difluoride with aminopropyl groups. The aminopropyl groups were protected with the DDZ group by reaction of the carbonyl chloride with the amino groups, a reaction readily known to those of skill in the art. The surface bearing these groups was placed in a solution of THF and contacted with a mask bearing a checkerboard pattern of 1 mm opaque and transparent regions. The mask was exposed to ultraviolet light having a wavelength down to at least about 280 nm for about 5 minutes at ambient temperature, although a wide range of exposure times and temperatures may be appropriate in various embodiments of the invention. For example, in one embodiment, an exposure time of between about 1 and 5000 seconds may be used at process temperatures of between −70 and +50° C.

In one preferred embodiment, exposure times of between about 1 and 500 seconds at about ambient pressure are used. In some preferred embodiments, pressure above ambient is used to prevent evaporation.

The surface of the membrane was then washed for about 1 hour with a fluorescent label which included an active ester bound to a chelate of a lanthanide. Wash times will vary over a wide range of values from about a few minutes to a few hours. These materials fluoresce in the red and the green visible region. After the reaction with the active ester in the fluorophore was complete, the locations in which the fluorophore was bound could be visualized by exposing them to ultraviolet light and observing the red and the green fluorescence. It was observed that the derivatized regions of the substrate closely corresponded to the original pattern of the mask.

D. Demonstration of Signal Capability

Signal detection capability was demonstrated using a low-level standard fluorescent bead kit manufactured by Flow Cytometry Standarda and having model no. 824. This kit includes 5.8 μm diameter beads, each impregnated with a known number of fluorescein molecules.

One of the beads was placed in the illumination field on the scan stage as shown in FIG. **9** in a field of a laser spot which was initially shuttered. After being positioned in the illumination field, the photon detection equipment was turned on. The laser beam was unblocked and it interacted with the particle bead, which then fluoresced. Fluorescence curves of beads impregnated with 7,000; 13,000; and 29,000 fluorescein molecules, are shown in FIGS. **11**A, **11**B, and **11**C respectively. On each curve, traces for beads without fluorescein molecules are also shown. These experiments were performed with 488 nm excitation, with 100 μW of laser power. The light was focused through a 40 power 0.75 NA objective.

The fluorescence intensity in all cases started off at a high value and then decreased exponentially. The fall-off in intensity is due to photobleaching of the fluorescein molecules. The traces of beads without fluorescein molecules are used for background subtraction. The difference in the initial exponential decay between labeled and nonlabeled beads is integrated to give the total number of photon counts, and this number is related to the number of molecules per bead. Therefore, it is possible to deduce the number of photons per fluorescein molecule that can be detected. For the curves illustrated in FIG. **11**, this calculation indicates the radiation of about 40 to 50 photons per fluorescein molecule are detected.

E. Determination of the Number of Molecules Per Unit Area

Aminopropylated glass microscope slides prepared according to the methods discussed above were utilized in

order to establish the density of labeling of the slides. The free amino termini of the slides were reacted with FITC (fluorescein isothiocyanate) which forms a covalent linkage with the amino group. The slide is then scanned to count the number of fluorescent photons generated in a region which, using the estimated 40–50 photons per fluorescent molecule, enables the calculation of the number of molecules which are on the surface per unit area.

A slide with aminopropyl silane on its surface was immersed in a 1 mM solution of FITC in DMF for 1 hour at about ambient temperature. After reaction, the slide was washed twice with DMF and then washed with ethanol, water, and then ethanol again. It was then dried and stored in the dark until it was ready to be examined.

Through the use of curves similar to those shown in FIG. **11**, and by integrating the fluorescent counts under the exponentially decaying signal, the number of free amino groups on the surface after derivitization was determined. It was determined that slides with labeling densities of 1 fluoroscein per $10^3 \times 10^3$ to ~2×2 nm could be reproducibly made as the concentration of aminopropyltriethoxysilane varied from 10^{-5}% to 10^{-1}%.

F. Removal of NVOC and Attachment of A Fluorescent Marker

NVOC-GABA groups were attached as described above. The entire surface of one slide was exposed to light so as to expose a free amino group at the end of the gamma amino butyric acid. This slide, and a duplicate which was not exposed, were then exposed to fluorescein isothiocyanate (FITC).

FIG. **12A** illustrates the slide which was not exposed to light, but which was exposed to FITC. The units of the x axis are time and the units of the y axis are counts. The trace contains a certain amount of background fluorescence. The duplicate slide was exposed to 350 nm broadband illumination for about 1 minute (12 mW/cm^2, ~350 nm illumination), washed and reacted with FITC. The fluorescence curves for this slide are shown in FIG. **12B**. A large increase in the level of fluorescence is observed, which indicates photolysis has exposed a number of amino groups on the surface of the slides for attachment of a fluorescent marker.

G. Use of a Mask in Removal of NVOC

The next experiment was performed with a 0.1% aminopropylated slide. Light from a Hg-Xe arc lamp was imaged onto the substrate through a laser-ablated chrome-on-glass mask in direct contact with the substrate.

This slide was illuminated for approximately 5 minutes, with 12 mW of 350 nm broadband light and then reacted with the 1 mM FITC solution. It was put on the laser detection scanning stage and a graph was plotted as a two-dimensional representation of position color-coded for fluorescence intensity. The fluorescence intensity (in counts) as a function of location is given on the color scale to the right of FIG. **13A** for a mask having 100×100 μm squares.

The experiment was repeated a number of times through various masks. The fluorescence pattern for a 50 μm mask is illustrated in FIG. **13B**, for a 20 μm mask in FIG. **13C**, and for a 10 μm mask in FIG. **13D**. The mask pattern is distinct down to at least about 10 μm squares using this lithographic technique.

H. Attachment of YGGFL and Subsequent Exposure to Herz Antibody and Goat Antimouse

In order to establish that receptors to a particular polypeptide sequence would bind to a surface-bound peptide and be detected, Leu enkephalin was coupled to the surface and recognized by an antibody. A slide was derivatized with 0.1% amino propyl-triethoxysilane and protected with NVOC. A 500 μm checkerboard mask was used to expose the slide in a flow cell using backside contact printing. The Leu enkephalin sequence (H_2N-tyrosine, glycine, glycine, phenylalanine, leucine-CO_2H, otherwise referred to herein as YGGFL) was attached via its carboxy end to the exposed amino groups on the surface of the slide. The peptide was added in DMF solution with the BOP/HOBT/DIEA coupling reagents and recirculated through the flow cell for 2 hours at room temperature.

A first antibody, known as the Herz antibody, was applied to the surface of the slide for 45 minutes at 2 μg/ml in a supercocktail (containing 1% BSA and 1% ovalbumin also in this case). A second antibody, goat anti-mouse fluorescein conjugate, was then added at 2 μg/ml in the supercocktail buffer, and allowed to incubate for 2 hours.

The results of this experiment are provided in FIG. **14**. Again, this figure illustrates fluorescence intensity as a function of position. The fluorescence scale is shown on the right, according to the color coding. This image was taken at 10 μm steps. This figure indicates that not only can deprotection be carried out in a well defined pattern, but also that (1) the method provides for successful coupling of peptides to the surface of the substrate, (2) the surface of a bound peptide is available for binding with an antibody, and (3) that the detection apparatus capabilities are sufficient to detect binding of a receptor.

I. Monomer-by-Monomer Formation of YGGFL and Subsequent Exposure to Labeled Antibody

Monomer-by-monomer synthesis of YGGFL and GGFL in alternate squares was performed on a slide in a checkerboard pattern and the resulting slide was exposed to the Herz antibody. This experiment and the results thereof are illustrated in FIGS. **15A**, **15B**, **15C**, and **15D**.

In FIG. **15A**, a slide is shown which is derivatized with the aminopropyl group, protected in this case with t-BOC (t-butoxycarbonyl). The slide was treated with TFA to remove the t-BOC protecting group. E-aminocaproic acid, which was t-BOC protected at its amino group, was then coupled onto the aminopropyl groups. The aminocaproic acid serves as a spacer between the aminopropyl group and the peptide to be synthesized. The amino end of the spacer was deprotected and coupled to NVOC-leucine. The entire slide was then illuminated with 12 mW of 325 nm broadband illumination. The slide was then coupled with NVOC-phenylalanine and washed. The entire slide was again illuminated, then coupled to NVOC-glycine and washed. The slide was again illuminated and coupled to NVOC-glycine to form the sequence shown in the last portion of FIG. **15A**.

As shown in FIG. **15B**, alternating regions of the slide were then illuminated using a projection print using a 500×500 μm checkerboard mask; thus, the amino group of glycine was exposed only in the lighted areas. When the next coupling chemistry step was carried out, NVOC-tyrosine was added, and it coupled only at those spots which had received illumination. The entire slide was then illuminated to remove all the NVOC groups, leaving a checkerboard of YGGFL in the lighted areas and in the other areas, GGFL. The Herz antibody (which recognizes the YGGFL, but not GGFL) was then added, followed by goat anti-mouse fluorescein conjugate.

The resulting fluorescence scan is shown in FIG. **15C**, and the color coding for the fluorescence intensity is again given on the right. Dark areas contain the tetrapeptide GGFL, which is not recognized by the Herz antibody (and thus there is no binding of the goat anti-mouse antibody with fluores-

cein conjugate), and in the red areas YGGFL is present. The YGGFL pentapeptide is recognized by the Herz antibody and, therefore, there is antibody in the lighted regions for the fluorescein-conjugated goat anti-mouse to recognize.

Similar patterns are shown for a 50 μm mask used in direct contact ("proximity print") with the substrate in FIG. **15**D. Note that the pattern is more distinct and the corners of the checkerboard pattern are touching when the mask is placed in direct contact with the substrate (which reflects the increase in resolution using this technique).

J. Monomer-by-Monomer Synthesis of YGGFL and PGGFL

A synthesis using a 50 μm checkerboard mask similar to that shown in FIG. **15** was conducted. However, P was added to the GGFL sites on the substrate through an additional coupling step. P was added by exposing protected GGFL to light through a mask, and subsequence exposure to P in the manner set forth above. Therefore, half of the regions on the substrate contained YGGFL and the remaining half contained PGGFL.

The fluorescence plot for this experiment is provided in FIG. **16**. As shown, the regions are again readily discernable. This experiment demonstrates that antibodies are able to recognize a specific sequence and that the recognition is not length-dependent.

K. Monomer-by-Monomer Synthesis of YGGFL and YPGGFL

In order to further demonstrate the operability of the invention, a 50 μm checkerboard pattern of alternating YGGFL and YPGGFL was synthesized on a substrate using techniques like those set forth above. The resulting fluorescence plot is provided in FIG. **17**. Again, it is seen that the antibody is clearly able to recognize the YGGFL sequence and does not bind significantly at the YPGGFL regions.

L. Synthesis of an Array of Sixteen Different Amino Acid Sequences and Estimation of Relative Binding Affinity to Herz Antibody

Using techniques similar to those set forth above, an array of 16 different amino acid sequences (replicated four times) was synthesized on each of two glass substrates. The sequences were synthesized by attaching the sequence NVOC-GFL across the entire surface of the slides. Using a series of masks, two layers of amino acids were then selectively applied to the substrate. Each region had dimensions of 0.25 cm×0.0625 cm. The first slide contained amino acid sequences containing only L amino acids while the second slide contained selected D amino acids. FIGS. **18**A and **18**B illustrate a map of the various regions on the first and second slides, respectively. The patterns shown in FIGS. **18**A and **18**B were duplicated four times on each slide. The slides were then exposed to the Herz antibody and fluorescein-labeled goat anti-mouse.

FIG. **19** is a fluorescence plot of the first slide, which contained only L amino acids. Red indicates strong binding (149,000 counts or more) while black indicates little or no binding of the Herz antibody (20,000 counts or less). The bottom right-hand portion of the slide appears "cut off" because the slide was broken during processing. The sequence YGGFL is clearly most strongly recognized. The sequences YAGFL and YSGFL also exhibit strong recognition of the antibody. By contrast, most of the remaining sequences show little or no binding. The four duplicate portions of the slide are extremely consistent in the amount of binding shown therein.

FIG. **20** is a fluorescence plot of the second slide. Again, strongest binding is exhibited by the YGGFL sequence. Significant binding is also detected to YaGFL, YsGFL, and YpGFL. The remaining sequences show less binding with the antibody. Note the low binding efficiency of the sequence yGGFL.

Table 6 lists the various sequences tested in order of relative fluorescence, which provides information regarding relative binding affinity.

TABLE 6

Apparent Binding to Herz Ab	
L-a.a. Set	D-a.a. Set
YGGFL	YGGFL
YAGFL	YaGFL
YSGFL	YsGFL
LGGFL	YpGFL
FGGFL	fGGFL
YPGFL	yGGFL
LAGFL	faGFL
FAGFL	wGGFL
WGGFL	yaGFL
	fpGFL
	waGFL

VIII. Illustrative Alternative Embodiment

According to an alternative embodiment of the invention, the methods provide for attaching to the surface a caged binding member which in its caged form has a relatively low affinity for other potentially binding species, such as receptors and specific binding substances. Such techniques are more fully described in copending application Ser. No. 404,920, filed Sep. 8, 1989, and incorporated herein by reference for all purposes.

According to this alternative embodiment, the invention provides methods for forming predefined regions on a surface of a solid support, wherein the predefined regions are capable of immobilizing receptors. The methods make use of caged binding members attached to the surface to enable selective activation of the predefined regions. The caged binding members are liberated to act as binding members ultimately capable of binding receptors upon selective activation of the predefined regions. The activated binding members are then used to immobilize specific molecules such as receptors on the predefined region of the surface. The above procedure is repeated at the same or different sites on the surface so as to provide a surface prepared with a plurality of regions on the surface containing, for example, the same or different receptors. When receptors immobilized in this way have a differential affinity for one or more ligands, screenings and assays for the ligands can be conducted in the regions of the surface containing the receptors.

The alternative embodiment may make use of novel caged binding members attached to the substrate. Caged (unactivated) members have a relatively low affinity for receptors of substances that specifically bind to uncaged binding members when compared with the corresponding affinities of activated binding members. Thus, the binding members are protected from reaction until a suitable source of energy is applied to the regions of the surface desired to be activated. Upon application of a suitable energy source, the caging groups labilize, thereby presenting the activated binding member. A typical energy source will be light.

Once the binding members on the surface are activated they may be attached to a receptor. The receptor chosen may be a monoclonal antibody, a nucleic acid sequence, a drug receptor, etc. The receptor will usually, though not always, be prepared so as to permit attaching it, directly or indirectly, to a binding member. For example, a specific binding substance having a strong binding affinity for the binding

member and a strong affinity for the receptor or a conjugate of the receptor may be used to act as a bridge between binding members and receptors if desired. The method uses a receptor prepared such that the receptor retains its activity toward a particular ligand.

Preferably, the caged binding member attached to the solid substrate will be a photoactivatable biotin complex, i.e., a biotin molecule that has been chemically modified with photoactivatable protecting groups so that it has a significantly reduced binding affinity for avidin or avidin analogs than does natural biotin. In a preferred embodiment, the protecting groups localized in a predefined region of the surface will be removed upon application of a suitable source of radiation to give binding members, that are biotin or a functionally analogous compound having substantially the same binding affinity for avidin or avidin analogs as does biotin.

In another preferred embodiment, avidin or an avidin analog is incubated with activated binding members on the surface until the avidin binds strongly to the binding members. The avidin so immobilized on predefined regions of the surface can then be incubated with a desired receptor or conjugate of a desired receptor. The receptor will preferably be biotinylated, e.g., a biotinylated antibody, when avidin is immobilized on the predefined regions of the surface. Alternatively, a preferred embodiment will present an avidin/biotinylated receptor complex, which has been previously prepared, to activated binding members on the surface.

IX. Conclusion

The present inventions provide greatly improved methods and apparatus for synthesis of polymers on substrates. It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reviewing the above description. By way of example, the invention has been described primarily with reference to the use of photoremovable protective groups, but it will be readily recognized by those of skill in the art that sources of radiation other than light could also be used. For example, in some embodiments it may be desirable to use protective groups which are sensitive to electron beam irradiation, x-ray irradiation, in combination with electron beam lithograph, or x-ray lithography techniques. Alternatively, the group could be removed by exposure to an electric current. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

What is claimed is:

1. A polynucleotide analysis apparatus comprising:

a capillary substrate having a surface that is made of a material that is different from the remainder of the capillary, said surface comprising a plurality of different polynucleotides, each of said different polynucleotides occupying a different area of said surface, each of said areas being 10^{-1} cm^2 or less in detectable area, said surface being within the capillary and comprising more than 10 of such polynucleotides, at least some of said polynucleotides bearing fluorescently labeled target molecules excitable with a laser;

an argon laser light source capable of generating light at a wavelength of about 488 nm or less at said surface to excite said fluorescently labeled target molecules, which generate light at a frequency greater than 488 nm;

said laser oriented to direct the laser light at said surface at an angle capable of contacting said target molecules;

a CCD detector oriented in a manner capable of detecting light fluoresced from said target molecules in said areas; and

a data collection, recording and management system for storing fluoresced light intensity, said data collection, recording and management system coupled to said CCD detector, said data collection, recording and management system storing light intensity and converting said light intensity into nucleic acid sequence information.

2. A polynucleotide analysis apparatus in accordance with claim **1** wherein the substrate is translated relative to the laser light.

3. A polynucleotide analysis apparatus in accordance with claim **1** wherein the capillary is composed of quartz.

4. A polynucleotide analysis apparatus in accordance with claim **1** wherein the surface is a gel.

5. A polynucleotide analysis apparatus in accordance with claim **4** wherein the apparatus is used in DNA gel scanning.

6. A polynucleotide analysis apparatus in accordance with claim **4** wherein there are a plurality of capillaries.

7. A polynucleotide analysis apparatus comprising an argon laser light source capable of generating a laser beam providing light at a wavelength of about 488 nm or less to excite fluorescently labeled target molecules attached to said polynucleotides in a substrate to emit excited fluorescent light at a wavelength greater than 488 nm detected by a CCD detector oriented in a manner capable of detecting said emitted fluorescent light from said target molecules in said substrate irradiated by said laser beam, said substrate having more than 10 polynucleotides and each detectable polynucleotide being present in 10^{-1} cm^2 or less in detectable area, said CCD detector coupled to a data collection system for analyzing fluoresced light intensity and converting said light intensity into nucleic acid sequence information.

8. A polynucleotide analysis apparatus in accordance with claim **7** wherein the substrate is a plurality of capillaries.

9. A polynucleotide analysis apparatus in accordance with claim **8** wherein the substrate further comprises a gel or polymer.

10. A polynucleotide analysis apparatus in accordance with claim **8** wherein said apparatus is used for DNA or protein gel scanning.

11. A polynucleotide analysis apparatus in accordance with claim **8** further comprising a translation system including a translation table arranged to support said substrate.

12. A polynucleotide analysis apparatus in accordance with claim **11** including a lens system arranged to focus said laser beam onto said substrate.

13. A polynucleotide analysis apparatus in accordance with claim **8** wherein said fluorescently labeled target molecules are within the capillaries.

14. An apparatus for analyzing nucleic acid binding, comprising:

a substrate that comprises at least 1000 different spheres, beads, or particles having different species of nucleic acids attached thereto, the area of the substrate containing the at least 1000 spheres, beads, or particles being less than 1 cm2, at least some of the nucleic acids being bound to fluorescently labeled target nucleic acids;

a laser energy source to illuminate the fluorescent labels;

a detector to detect a fluorescent label bound to said target nucleic acids; and

a data collection system for storing fluoresced light intensity.

15. An apparatus in accordance with claim 14 wherein the substrate comprises wells, trenches or etched regions.

16. An apparatus in accordance with claim 14 wherein the detector comprises a microscope.

17. An apparatus in accordance with claim 14 wherein the detector comprises a CCD camera.

18. An apparatus in accordance with claim 14 wherein the wavelength of the laser is 488 nanometers or less.

19. An apparatus in accordance with claim 14 wherein the comprises beads.

20. An apparatus in accordance with claim 14 wherein the substrate comprises spheres.

21. An apparatus in accordance with claim 14 wherein the substrate comprises particles.

22. An apparatus in accordance with claim 18 wherein the substrate or its surface may be composed of a polymer, plastic, a resin, silica or silica-based materials, carbon, metals, or inorganic glasses.

23. An apparatus in accordance with claim 18 wherein the substrate or its surface may be composed of a polymer.

24. An apparatus in accordance with claim 18 wherein the substrate or its surface may be composed of silica.

25. An apparatus in accordance with claim 14 wherein the apparatus comprises a translator to move the substrate relative to the detector.

26. An apparatus in accordance with claim 14 wherein there are 10,000 different spheres, beads, or particles.

27. An apparatus for detecting binding of nucleic acids; comprising:

(a) a substrate that comprises at least 1000 different spheres, beads, or particles having different species of nucleic acids attached thereto, the area of the substrate containing the at least 1000 spheres, beads, or particles being less than 1 cm2, at least some of the nucleic acids being bound to fluorescently labeled target nucleic acids

(b) a laser excitation light source;

(c) a detector capable of receiving a signal from the fluorescent labels, the detector comprising a microscope;

(d) a translator to move the substrate relative to the detector; and

(e) a data collection system adapted to receive input from the detector.

28. An apparatus in accordance with claim 27 wherein the detector acquires data every 1 to 100 microns.

29. An apparatus in accordance with claim 27 wherein the detector acquires data every 0.8 to 10 microns.

30. An apparatus in accordance with claim 27 wherein the substrate comprises beads.

31. An apparatus in accordance with claim 27 wherein the substrate comprises spheres.

32. An apparatus in accordance with claim 27 wherein the substrate comprises particles.

33. An apparatus in accordance with claim 27 wherein the substrate or its surface may be composed of a polymer, plastic, a resin, silica or silica-based materials, carbon, metals, or inorganic glasses.

34. An apparatus in accordance with claim 27 wherein there are at least 10,000 different spheres, beads, or particles.

35. A method for screening large numbers of biological polymers, comprising:

providing target nucleic acids;

providing a substrate having an array of at least 1000 different beads, the different beads occupying an area on a substrate of less than 1 cm2, at least some of the different beads having different nucleic acids covalently attached thereto;

contacting the target nucleic acids and the beads so that after contact at least some of the nucleic acids on the beads hybridize to the target nucleic acids further comprising having fluorescently labeled nucleic acids bound thereto;

illuminating the array with a laser energy source to excite the fluorescent labels; and

detecting fluoresced light with a detector that is connected to a data storage system; and

determining which nucleic acids on the beads have bound to target nucleic acids.

36. A method in accordance with claim 35 wherein the detector comprises a microscope.

37. A method in accordance with claim 35 wherein the detector comprises a CCD camera.

38. A method in accordance with claim 35 wherein the substrate or its surface may be composed of a polymer, plastic, a resin, silica or silica-based materials, carbon, metals, or inorganic glasses.

39. A method in accordance with claim 35 wherein the fluorescent label is attached to the target nucleic acid before contact with the beads.

40. A method in accordance with claim 35 wherein the nucleic acids bound to the beads are oligonucleotides.

41. A method in accordance with claim 35 wherein there are 100,000 beads.

42. A method in accordance with claim 35 wherein the detector acquires data every 1 to 100 microns.

43. A method in accordance with claim 35 wherein the detector acquires data every 0.8 to 10 microns.

44. An apparatus in accordance with claim 14 wherein the detector acquires data every 1 to 100 microns.

45. An apparatus in accordance with claim 14 wherein the detector acquires data every 0.8 to 10 microns.

46. A method in accordance with claim 35 comprising providing 100,000 beads.

47. A method in accordance with claim 43 comprising providing 100,000 beads.

48. An apparatus in accordance with claim 27 wherein the area of the substrate is less than 10^{-1} cm^2.

49. A method in accordance with claim 35 wherein the area of the substrate is less than 10^{-1} cm^2.

50. An apparatus in accordance wit claim 27 wherein the area of the substrate is less than 10^{-1} cm^2.

51. A polynucleotide analysis apparatus in accordance with claim 6 wherein there are greater than 100 polynucleotides within the capillaries.

52. An apparatus for detecting binding of nucleic acids; comprising:

(a) a substrate that comprises and at least 1000 different spheres, beads, or particles having different species of nucleic acids attached thereto, the area of the substrate containing the at least 1000 spheres, beads, or particles being less than 1 cm^2, at least some of the nucleic acids being fluorescently labeled;

(b) an excitation light source;

(c) a CCD detector capable of receiving a signal from the fluorescent labels,

(d) a data collection system adapted to receive input from the detector.

53. A method for screening large numbers of biological polymers, comprising:

providing target nucleic acids;

providing a substrate having an array of at least 1000 different beads, the different beads occupying an area

on a substrate of less than 1 cm^2, at least some of the different beads having different nucleic acids covalently attached thereto;

contacting the target nucleic acids and the beads so that after contact at least some of the nucleic acids on the beads hybridize to the target nucleic acids further comprising having fluorescently labeled nucleic acids bound thereto;

illuminating the array with an energy source to excite the fluorescent labels; and

detecting fluoresced light with a CCD detector that is connected to a data storage system; and

determining which nucleic acids on the beads have bound to target nucleic acids.

* * * * *

EXHIBIT 8

US005545531A

United States Patent [19]

Rava et al.

[11] Patent Number: 5,545,531

[45] Date of Patent: Aug. 13, 1996

[54] **METHODS FOR MAKING A DEVICE FOR CONCURRENTLY PROCESSING MULTIPLE BIOLOGICAL CHIP ASSAYS**

[75] Inventors: **Richard P. Rava**, San Jose; **Stephen P. A. Fodor**, Palo Alto; **Mark Trulson**, San Jose, all of Calif.

[73] Assignee: **Affymax Technologies N.V.**, Palo Alto, Calif.

[21] Appl. No.: **476,850**

[22] Filed: **Jun. 7, 1995**

[51] **Int. Cl.**[6] **C12Q 1/68**

[52] **U.S. Cl.** **435/6**; 204/403; 935/78

[58] **Field of Search** 435/6, 78; 204/403

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,143,854	9/1992	Pirrung et al.	436/518
5,196,305	3/1993	Findlay et al.	935/78
5,288,514	2/1994	Ellman	427/2
5,348,855	9/1994	Dattagupta et al.	435/6
5,382,512	1/1995	Smethers et al.	435/6
5,384,261	1/1995	Winkler et al.	436/518

FOREIGN PATENT DOCUMENTS

WO90/15070	6/1990	WIPO .
WO92/10092	11/1991	WIPO .

OTHER PUBLICATIONS

Materials from HYSEQ Inc.: "Genome Sequencing Machine" p. 60; Summary materials–5 pgs.

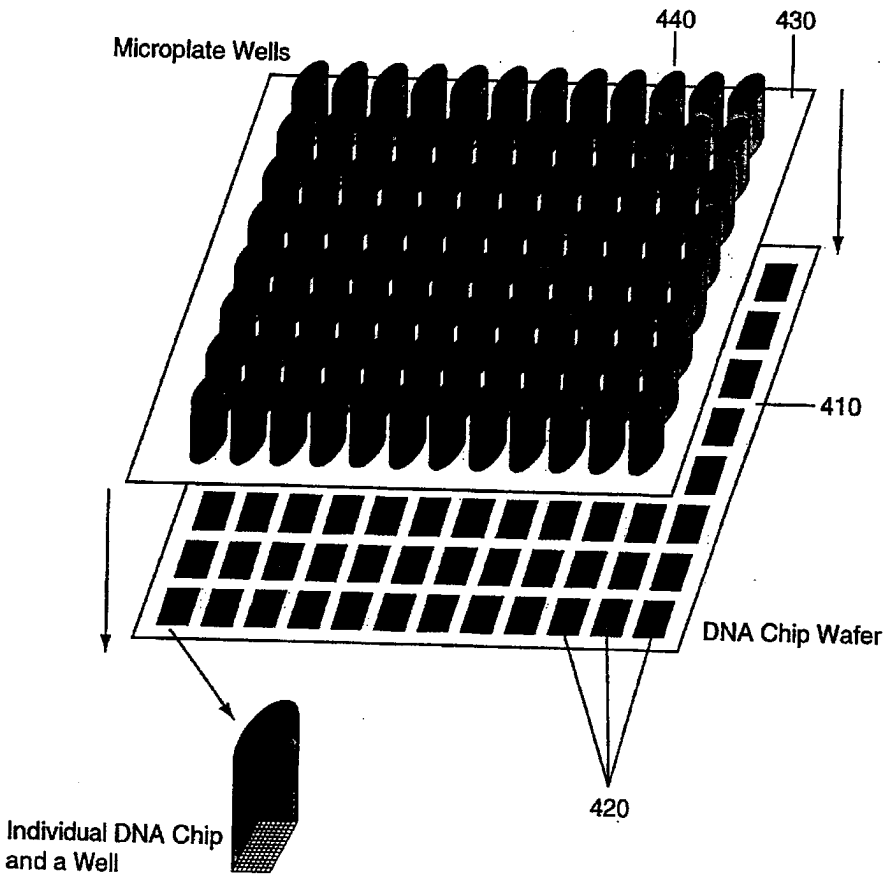
Primary Examiner—Paul B. Prebilic

Attorney, Agent, or Firm—Townsend and Townsend and Crew LLP

[57] **ABSTRACT**

Methods for concurrently processing multiple biological chip assays by providing a biological chip plate comprising a plurality of test wells, each test well having a biological chip having a molecular probe array; introducing samples into the test wells; subjecting the biological chip plate to manipulation by a fluid handling device that automatically performs steps to carry out reactions between target molecules in the samples and probes; and subjecting the biological chip plate to a biological chip plate reader that interrogates the probe arrays to detect any reactions between target molecules and probes.

4 Claims, 8 Drawing Sheets



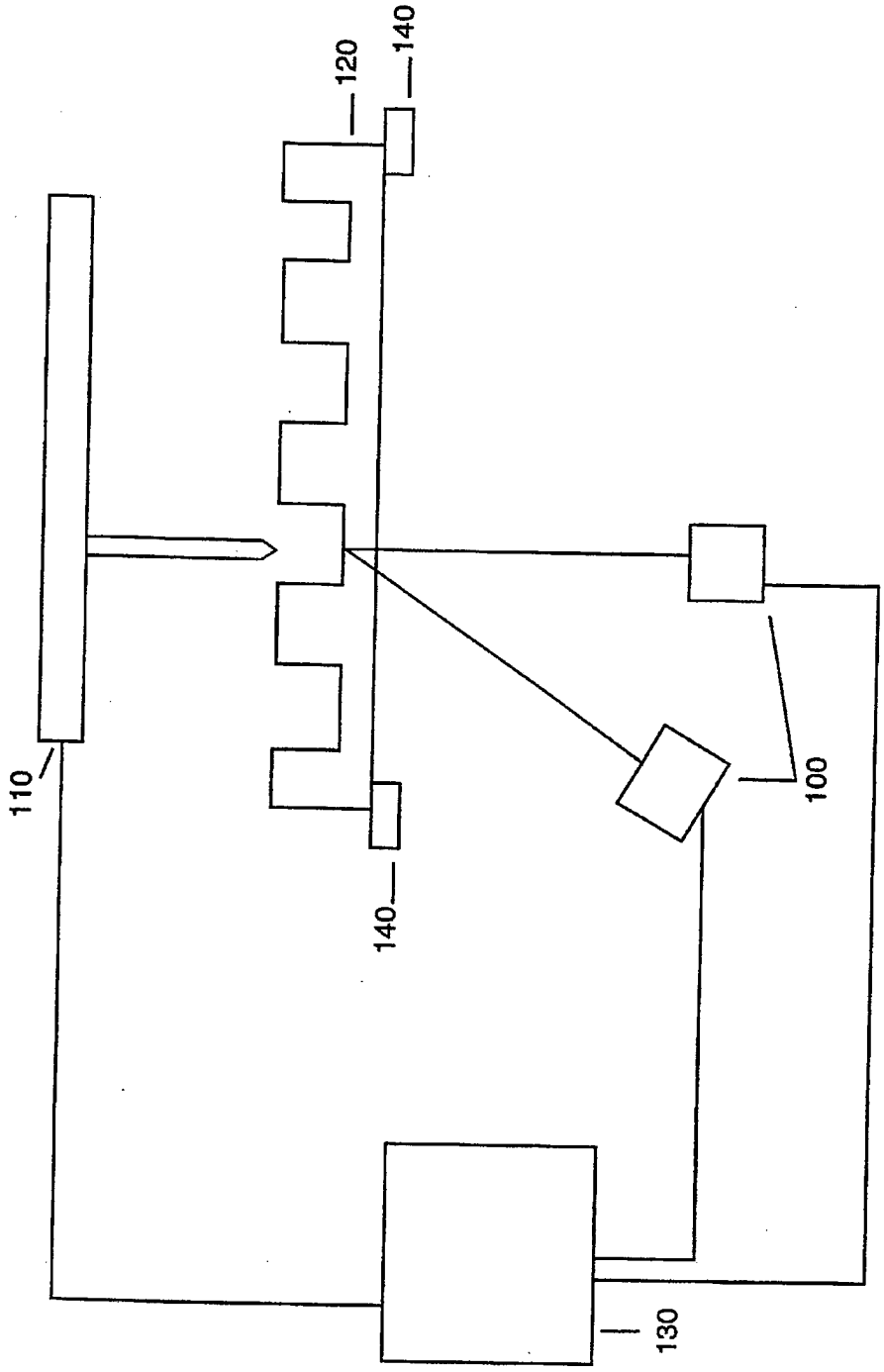


Figure 1

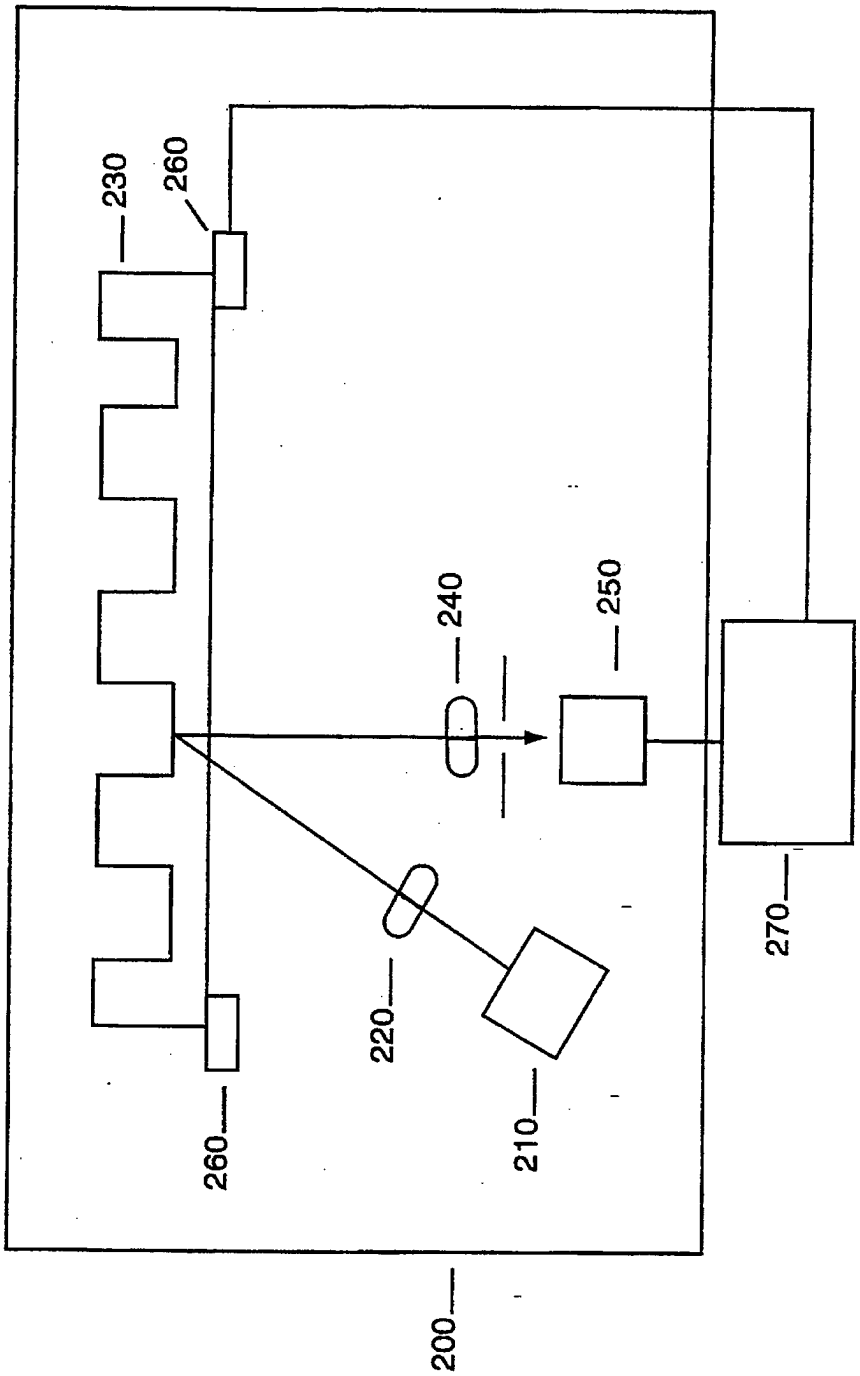


Figure 2

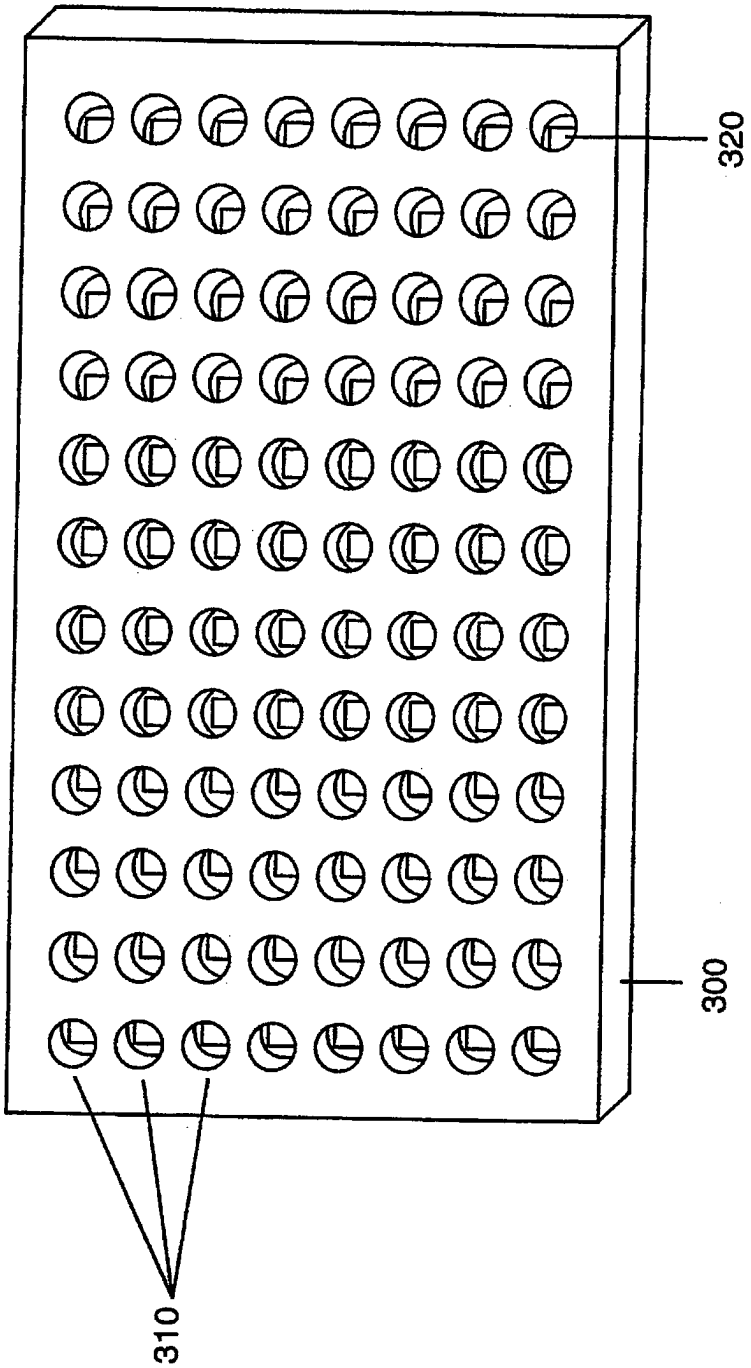


Figure 3

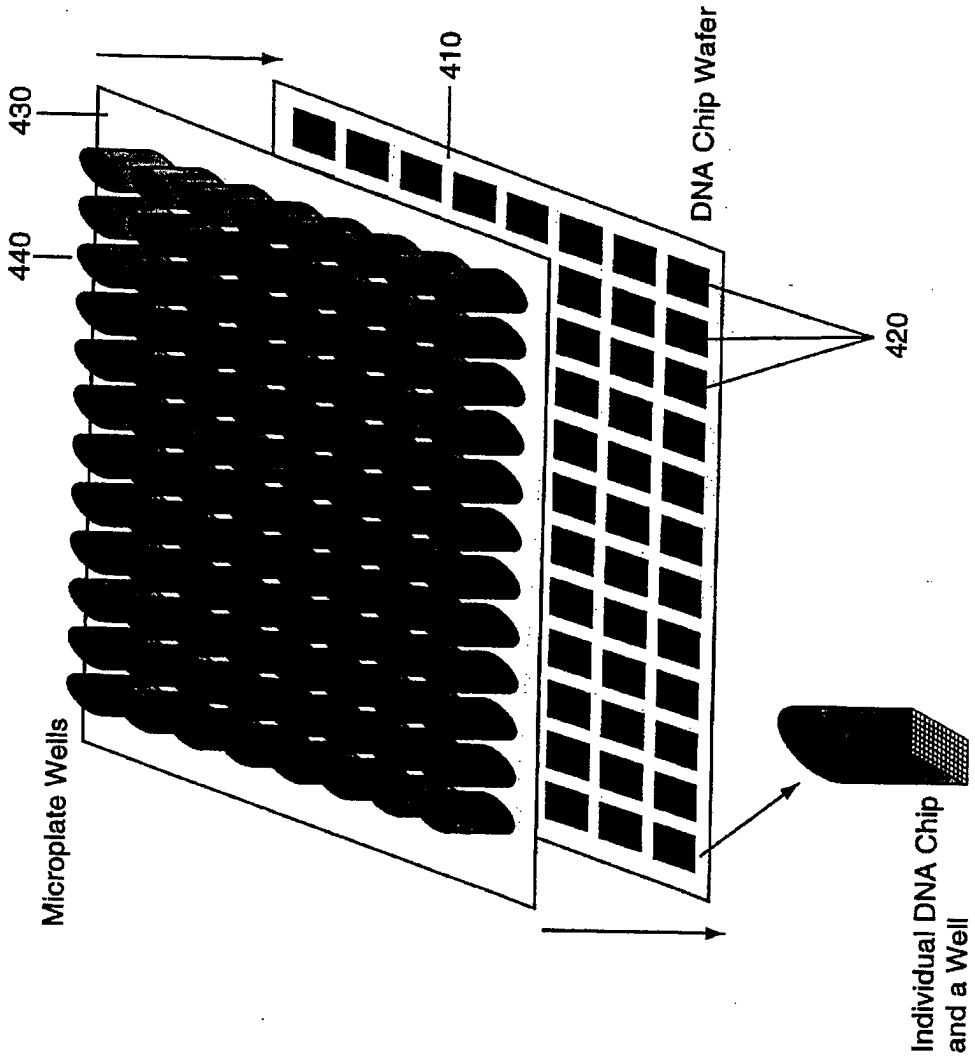

430
440
410
DNA Chip Wafer
420
Microplate Wells
Individual DNA Chip and a Well

Figure 4

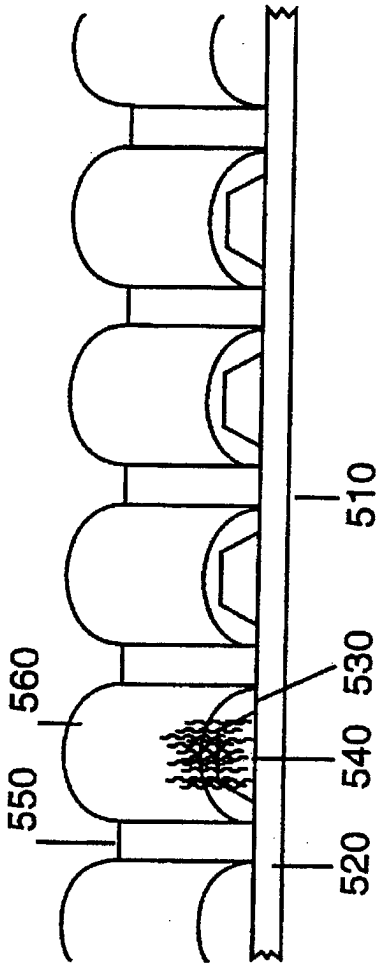


Figure 5

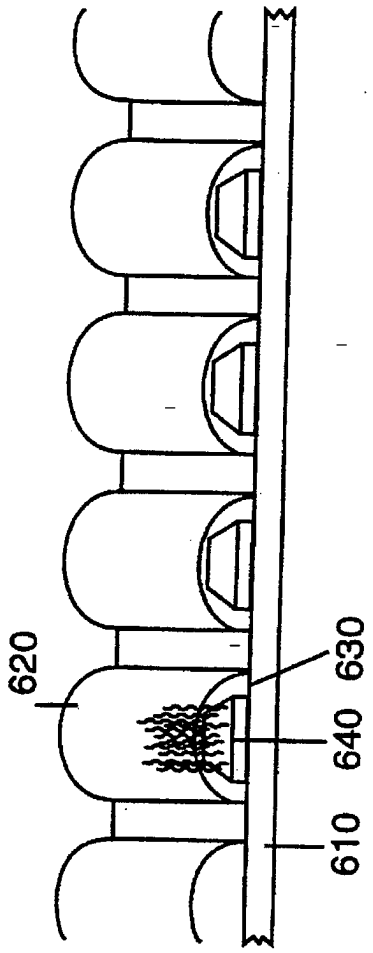


Figure 6

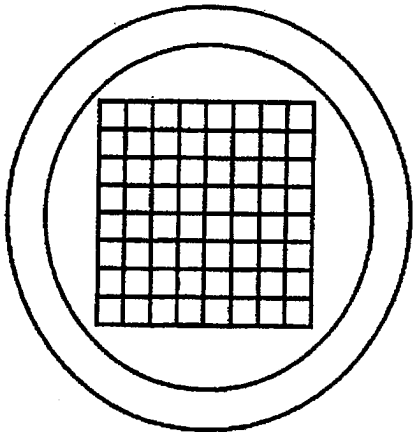


Figure 7

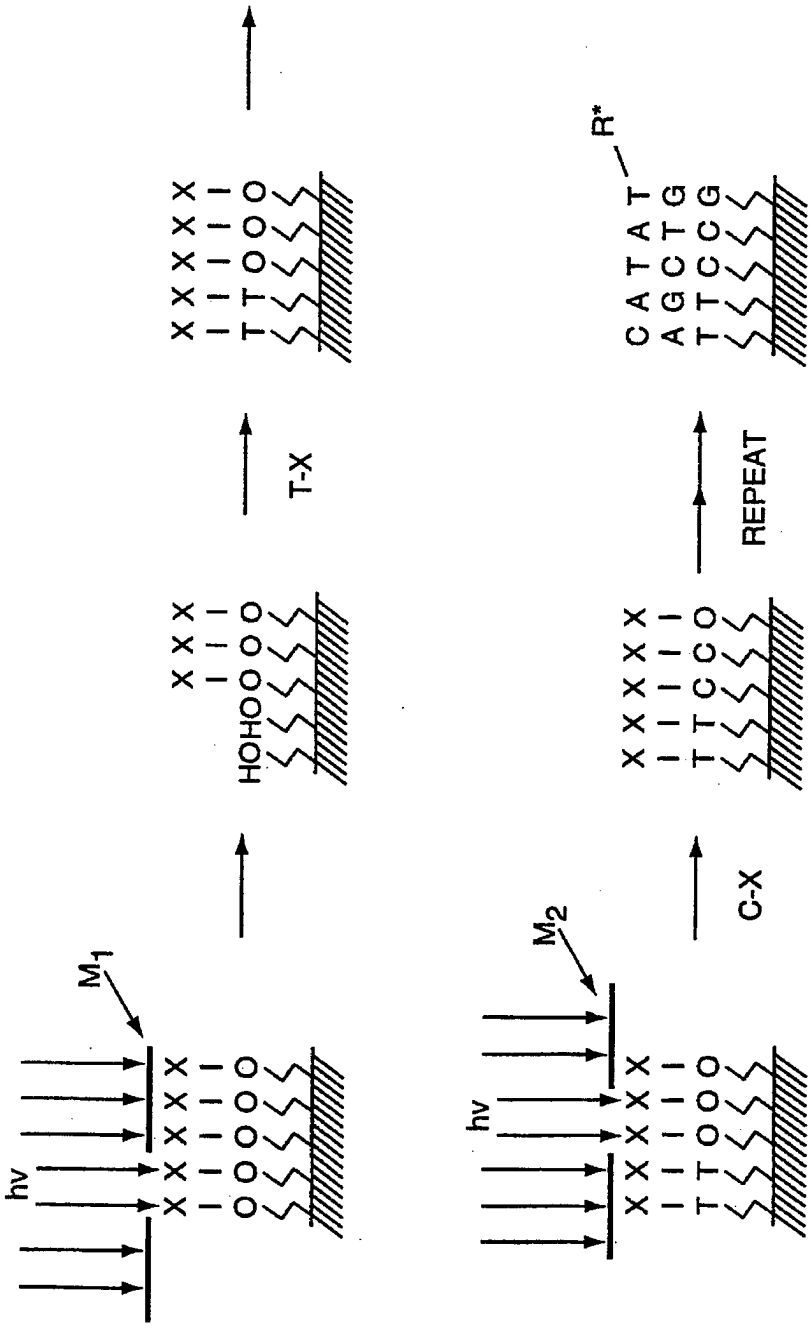


Figure 8

METHODS FOR MAKING A DEVICE FOR CONCURRENTLY PROCESSING MULTIPLE BIOLOGICAL CHIP ASSAYS

BACKGROUND OF THE INVENTION

This invention relates to methods for concurrently performing multiple biological chip assays. The invention therefore relates to diverse fields impacted by the nature of molecular interaction, including chemistry, biology, medicine and diagnostics.

New technology, called VLSIPS™, has enabled the production of chips smaller than a thumbnail that contain hundreds of thousands or more of different molecular probes. These biological chips or arrays have probes arranged in arrays, each probe assigned a specific location. Biological chips have been produced in which each location has a scale of, for example, ten microns. The chips can be used to determine whether target molecules interact with any of the probes on the chip. After exposing the array to target molecules under selected test conditions, scanning devices can examine each location in the array and determine whether a target molecule has interacted with the probe at that location.

Biological chips or arrays are useful in a variety of screening techniques for obtaining information about either the probes or the target molecules. For example, a library of peptides can be used as probes to screen for drugs. The peptides can be exposed to a receptor, and those probes that bind to the receptor can be identified.

Arrays of nucleic acid probes can be used to extract sequence information from, for example, nucleic acid samples. The samples are exposed to the probes under conditions that allow hybridization. The arrays are then scanned to determine to which probes the sample molecules have hybridized. One can obtain sequence information by careful probe selection and using algorithms to compare patterns of hybridization and non-hybridization. This method is useful for sequencing nucleic acids, as well as sequence checking. For example, the method is useful in diagnostic screening for genetic diseases or for the presence and/or identity of a particular pathogen or a strain of pathogen. For example, there are various strains of HIV, the virus that causes AIDS. Some of them have become resistant to current AIDS therapies. Diagnosticians can use DNA arrays to examine a nucleic acid sample from the virus to determine what strain it belongs to.

Currently, chips are treated individually, from the step of exposure to the target molecules to scanning. These methods yield exquisitely detailed information. However, they are not adapted for handling multiple samples simultaneously. The ability to do so would be advantageous in settings in which large amounts of information are required quickly, such as in clinical diagnostic laboratories or in large-scale undertakings such as the Human Genome Project.

SUMMARY OF THE INVENTION

This invention provides methods for concurrently processing multiple biological chip assays. According to the methods, a biological chip plate comprising a plurality of test wells is provided. Each test well defines a space for the introduction of a sample and contains a biological array. The array is formed on a surface of the substrate, with the probes exposed to the space. A fluid handling device manipulates the plates to perform steps to carry out reactions between the target molecules in samples and the probes in a plurality of test wells. The biological chip plate is then interrogated by a biological chip plate reader to detect any reactions between target molecules and probes in a plurality of the test wells, thereby generating results of the assay. In a further embodiment of the invention, the method also includes processing the results of the assay with a computer. Such analysis is useful when sequencing a gene by a method that uses an algorithm to process the results of many hybridization assays to provide the nucleotide sequence of the gene.

The methods of the invention can involve the binding of tagged target molecules to the probes. The tags can be, for example, fluorescent markers, chemiluminescent markers, light scattering markers or radioactive markers. In certain embodiments, the probes are nucleic acids, such as DNA or RNA molecules. The methods can be used to detect or identify a pathogenic organism, such as HIV, or to detect a human gene variant, such a the gene for a genetic disease such as cystic fibrosis, diabetes, muscular dystrophy or predisposition to certain cancers.

This invention also provides systems for performing the methods of this invention. The systems include a biological chip plate; a fluid handling device that automatically performs steps to carry out assays on samples introduced into a plurality of the test wells; a biological chip plate reader that determines in a plurality of the test wells the results of the assay and, optionally, a computer comprising a program for processing the results. The fluid handling device and plate reader can have a heater/cooler controlled by a thermostat for controlling the temperature of the samples in the test wells and robotically controlled pipets for adding or removing fluids from the test wells at predetermined times.

In certain embodiments, the probes are attached by light-directed probe synthesis. The biological chip plates can have 96 wells arranged in 8 rows and 12 columns, such as a standard microtiter plate. The probe arrays can each have at least about 100, 1000, 100,000 or 1,000,000 addressable features (e.g., probes). A variety of probes can be used on the plates, including, for example, various polymers such as peptides or nucleic acids.

The plates can have wells in which the probe array in each test well is the same. Alternatively, when each of several samples are to be subjected to several tests, each row can have the same probe array and each column can have a different array. Alternatively, all the wells can have different arrays.

Several methods of making biological chip plates are contemplated. In one method, a wafer and a body are provided. The wafer includes a substrate and a surface to which is attached a plurality of arrays of probes. The body has a plurality of channels. The body is attached to the surface of the wafer whereby the channels each cover an array of probes and the wafer closes one end of a plurality of the channels, thereby forming test wells defining spaces for receiving samples. In a second method, a body having a plurality of wells defining spaces is provided and biological chips are provided. The chips are attached to the wells so that the probe arrays are exposed to the space. Another embodiment involves providing a wafer having a plurality of probe arrays; and applying a material resistant to the flow of a liquid sample so as to surround the probe arrays, thereby creating test wells.

This invention also provides a wafer for making a biological chip plate. The wafer has a substrate and a surface to which are attached a plurality of probe arrays. The probe arrays are arranged on the wafer surface in rows and columns, wherein the probe arrays in each row are the same and the probe arrays in each column are different.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts a system of this invention having a biological chip plate, fluid handling device, biological chip plate reader and computer;

FIG. **2** depicts the scanning of a biological chip plate by a biological chip plate reader;

FIG. **3** depicts a biological plate of this invention;

FIG. **4** depicts the mating of a wafer containing many biological arrays with a body having channels to create a biological chip plate;

FIG. **5** depicts a biological chip plate in cross section having a body attached to a wafer to create closed test wells in which a probe array is exposed to the space in the test well;

FIG. **6** depicts a biological plate in cross section having a body which has individual biological chips attached to the bottom of the wells;

FIG. **7** is a top-down view of a test well containing a biological array; and

FIG. **8** depicts a method of producing an array of oligonucleotide probes on the surface of a substrate by using a mask to expose certain parts of the surface to light, thereby removing photoremovable protective groups, and attaching nucleotides to the exposed reactive groups.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

The following terms are intended to have the following general meanings as they are used herein:

A. Complementary: Refers to the topological compatibility or matching together of interacting surfaces of a probe molecule and its target. Thus, the target and its probe can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.

B. Probe: A probe is a surface-immobilized molecule that can be recognized by a particular target. Examples of probes that can be investigated by this invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones (e.g., opioid peptides, steroids, etc.), hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs, lectins, sugars, oligonucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.

C. Target: A molecule that has an affinity for a given probe. Targets may be naturally-occurring or man-made molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Targets may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of targets which can be employed by this invention include, but are not restricted to, antibodies, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells or other materials), drugs, oligonucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles. Targets are sometimes referred to in the art as anti-probes. As the term targets is used herein, no difference in meaning is intended. A "Probe Target Pair" is formed when two macromolecules have combined through molecular recognition to form a complex.

D. Array: A collection of probes, at least two of which are different, arranged in a spacially defined and physically addressable manner.

E. Biological Chip: A substrate having a surface to which one or more arrays of probes is attached. The substrate can be, merely by way of example, silicon or glass and can have the thickness of a glass microscope slide or a glass cover slip. Substrates that are transparent to light are useful when the method of performing an assay on the chip involves optical detection. As used herein, the term also refers to a probe array and the substrate to which it is attached that form part of a wafer.

F. Wafer: A substrate having a surface to which a plurality of probe arrays are attached. On a wafer, the arrays are physically separated by a distance of at least about a millimeter, so that individual chips can be made by dicing a wafer or otherwise physically separating the array into units having a probe array.

G. Biological Chip Plate: A device having an array of biological chips in which the probe array of each chip is separated from the probe array of other chips by a physical barrier resistant to the passage of liquids and forming an area or space, referred to as a "test well," capable of containing liquids in contact with the probe array.

II. General

This invention provides automated methods for concurrently processing multiple biological chip assays. Currently available methods utilize each biological chip assay individually. The methods of this invention allow many tests to be set up and processed together. Because they allow much higher throughput of test samples, these methods greatly improve the efficiency of performing assays on biological chips.

In the methods of this invention, a biological chip plate is provided having a plurality of test wells. Each test well includes a biological chip. Test samples, which may contain target molecules, are introduced into the test wells. A fluid handling device exposes the test wells to a chosen set of reaction conditions by, for example, adding or removing fluid from the wells, maintaining the liquid in the wells at predetermined temperatures, and agitating the wells as required, thereby performing the test. Then, a biological chip reader interrogates the probe arrays in the test wells, thereby obtaining the results of the tests. A computer having an appropriate program can further analyze the results from the tests.

Referring to FIG. **1**, one embodiment of the invention is a system for concurrently processing biological chip assays. The system includes a biological chip plate reader **100**, a fluid handling device **110**, a biological chip plate **120** and, optionally, a computer **130**. In operation, samples are placed in wells on the chip plate **120** with fluid handling device **110**. The plate optionally can be moved with a stage translation device **140**. Reader **100** is used to identify where targets in the wells have bound to complementary probes. The system operates under control of computer **130** which may optionally interpret the results of the assay.

A. Biological Chip Plate Reader

In assays performed on biological chips, detectably labeled target molecules bind to probe molecules. Reading the results of an assay involves detecting a signal produced by the detectable label. Reading assays on a biological chip plate requires a biological chip reader. Accordingly, locations at which target(s) bind with complementary probes can be identified by detecting the location of the label. Through knowledge of the characteristics/sequence of the probe

versus location, characteristics of the target can be determined. The nature of the biological chip reader depends upon the particular type of label attached to the target molecules.

The interaction between targets and probes can be characterized in terms of kinetics and thermodynamics. As such, it may be necessary to interrogate the array while in contact with a solution of labeled targets. In such systems, the detection system must be extremely selective, with the capacity to discriminate between surface-bound and solution-born targets. Also, in order to perform a quantitative analysis, the high-density of the probe sequences requires the system to have the capacity to distinguish between each feature site. The system also should have sensitivity to low signal and a large dynamic range.

In one embodiment, the chip plate reader includes a confocal detection device having a monochromatic or polychromatic light source, a focusing system for directing an excitation light from the light source to the substrate, a temperature controller for controlling the substrate temperature during a reaction, and a detector for detecting fluorescence emitted by the targets in response to the excitation light. The detector for detecting the fluorescent emissions from the substrate, in some embodiments, includes a photomultiplier tube. The location to which light is directed may be controlled by, for example, an x-y-z translation table. Translation of the x-y-z table, temperature control, and data collection are managed and recorded by an appropriately programmed digital computer.

Further details for methods of detecting fluorescently labelled materials on biological chips are provided in U.S. patent application Ser. No. 08/195,889, filed Feb. 10, 1994 and incorporated herein by reference.

FIG. **2** illustrates the reader according to one specific embodiment. The chip plate reader comprises a body **200** for immobilizing the biological chip plate. Excitation radiation, from an excitation source **210** having a first wavelength, passes through excitation optics **220** from below the array. The light passes through the chip plate since it is transparent to at least this wavelength of light. The excitation radiation excites a region of a probe array on the biological chip plate **230**. In response, labeled material on the sample emits radiation which has a wavelength that is different from the excitation wavelength. Collection optics **240**, also below the array, then collect the emission from the sample and image it onto a detector **250**, which can house a CCD array, as described below. The detector generates a signal proportional to the amount of radiation sensed thereon. The signals can be assembled to represent an image associated with the plurality of regions from which the emission originated.

According to one embodiment, a multi-axis translation stage **260** moves the biological chip plate to position different wells to be scanned, and to allow different probe portions of a probe array to be interrogated. As a result, a 2-dimensional image of the probe arrays in each well is obtained.

The biological chip reader can include auto-focusing feature to maintain the sample in the focal plane of the excitation light throughout the scanning process. Further, a temperature controller may be employed to maintain the sample at a specific temperature while it is being scanned. The multi-axis translation stage, temperature controller, auto-focusing feature, and electronics associated with imaging and data collection are managed by an appropriately programmed digital computer **270**.

In one embodiment, a beam is focused onto a spot of about 2 μm in diameter on the surface of the plate using, for example, the objective lens of a microscope or other optical means to control beam diameter. (See, e.g., U.S. patent application Ser. No. 08/195,889, supra.)

In another embodiment, fluorescent probes are employed in combination with CCD imaging systems. Details of this method are described in U.S. application Ser. No. 08/301,051, incorporated herein by reference in its entirely. In many commercially available microplate readers, typically the light source is placed above a well, and a photodiode detector is below the well. In the present invention, the light source can be replaced with a higher power lamp or laser. In one embodiment, the standard absorption geometry is used, but the photodiode detector is replaced with a CCD camera and imaging optics to allow rapid imaging of the well. A series of Raman holographic or notch filters can be used in the optical path to eliminate the excitation light while allowing the emission to pass to the detector. In a variation of this method, a fiber optic imaging bundle is utilized to bring the light to the CCD detector. In another embodiment, the laser is placed below the biological chip plate and light directed through the transparent wafer or base that forms the bottom of the biological chip plate. In another embodiment, the CCD array is built into the wafer of the biological chip plate.

The choice of the CCD array will depend on the number of probes in each biological array. If 2500 probes nominally arranged in a square (50×50) are examined, and 6 lines in each feature are sampled to obtain a good image, then a CCD array of 300×300 pixels is desirable in this area. However, if an individual well has 48,400 probes (220×220) then a CCD array with 1320×1320 pixels is desirable. CCD detectors are commercially available from, e.g., Princeton Instruments, which can meet either of these requirements.

In another embodiment, the detection device comprises a line scanner, as described in U.S. patent application Ser. No. 08/301,051, filed Sep. 2, 1994, incorporated herein by reference. Excitation optics focuses excitation light to a line at a sample, simultaneously scanning or imaging a strip of the sample. Surface bound labeled targets from the sample fluoresce in response to the light. Collection optics image the emission onto a linear array of light detectors. By employing confocal techniques, substantially only emission from the light's focal plane is imaged. Once a strip has been scanned, the data representing the 1-dimensional image are stored in the memory of a computer. According to one embodiment, a multi-axis translation stage moves the device at a constant velocity to continuously integrate and process data. Alternatively, galvometric scanners or rotating polyhedral mirrors may be employed to scan the excitation light across the sample. As a result, a 2-dimensional image of the sample is obtained.

In another embodiment, collection optics direct the emission to a spectrograph which images an emission spectrum onto a 2-dimensional array of light detectors. By using a spectrograph, a full spectrally resolved image of the sample is obtained.

The read time for a full microtiter plate will depend on the photophysics of the fluorophore (i.e. fluorescence quantum yield and photodestruction yield) as well as the sensitivity of the detector. For fluorescein, sufficient signal-to-noise to read a chip image with a CCD detector can be obtained in about 30 seconds using 3 mW/cm^2 and 488 nm excitation from an Ar ion laser or lamp. By increasing the laser power, and switching to dyes such as CY3 or CY5 which have lower photodestruction yields and whose emission more closely matches the sensitivity maximum of the CCD detector, one easily is able to read each well in less than 5 seconds.

Thus, an entire plate could be examined quantitatively in less than 10 minutes, even if the whole plate has over 4.5 million probes.

A computer can transform the data into another format for presentation. Data analysis can include the steps of determining, e.g., fluorescent intensity as a function of substrate position from the data collected, removing "outliers" (data deviating from a predetermined statistical distribution), and calculating the relative binding affinity of the targets from the remaining data. The resulting data can be displayed as an image with color in each region varying according to the light emission or binding affinity between targets and probes therein.

One application of this system when coupled with the CCD imaging system that speeds performance of the tests is to obtain results of the assay by examining the on- or off-rates of the hybridization. In one embodiment of this method, the amount of binding at each address is determined at several time points after the probes are contacted with the sample. The amount of total hybridization can be determined as a function of the kinetics of binding based on the amount of binding at each time point. Thus, it is not necessary to wait for equilibrium to be reached. The dependence of the hybridization rate for different oligonucleotides on temperature, sample agitation, washing conditions (e.g. pH, solvent characteristics, temperature) can easily be determined in order to maximize the conditions for rate and signal-to-noise. Alternative methods are described in Fodor et al., U.S. Pat. No. 5,324,633, incorporated herein by reference.

B. Fluid Handling Instruments and Assay Automation

Assays on biological arrays generally include contacting a probe array with a sample under the selected reaction conditions, optionally washing the well to remove unreacted molecules, and analyzing the biological array for evidence of reaction between target molecules the probes. These steps involve handling fluids. The methods of this invention automate these steps so as to allow multiple assays to be performed concurrently. Accordingly, this invention employs automated fluid handling systems for concurrently performing the assay steps in each of the test wells. Fluid handling allows uniform treatment of samples in the wells. Microtiter robotic and fluid-handling devices are available commercially, for example, from Tecan AG.

The plate is introduced into a holder in the fluid-handling device. This robotic device is programmed to set appropriate reaction conditions, such as temperature, add samples to the test wells, incubate the test samples for an appropriate time, remove unreacted samples, wash the wells, add substrates as appropriate and perform detection assays. The particulars of the reaction conditions depends upon the purpose of the assay. For example, in a sequencing assay involving DNA hybridization, standard hybridization conditions are chosen. However, the assay may involve testing whether a sample contains target molecules that react to a probe under a specified set of reaction conditions. In this case, the reaction conditions are chosen accordingly.

C. Biological Chip Plates

FIG. **3** depicts an example of a biological chip plate **300** used in the methods of this invention based on the standard 96-well microtiter plate in which the chips are located at the bottom of the wells. Biological chip plates include a plurality of test wells **310**, each test well defining an area or space for the introduction of a sample, and each test well comprising a biological chip **320**, i.e., a substrate and a surface to which an array of probes is attached, the probes being exposed to the space. FIG. **7** shows a top-down view of a well of a biological chip plate of this invention containing a biological chip on the bottom surface of the well.

This invention contemplates a number of embodiments of the biological chip plate. In a preferred embodiment, depicted in FIG. **4**, the biological chip plate includes two parts. One part is a wafer **410** that includes a plurality of biological arrays **420**. The other part is the body of the plate **430** that contains channels **440** that form the walls of the well, but that are open at the bottom. The body is attached to the surface of the wafer so as to close one end of the channels, thereby creating wells. The walls of the channels are placed on the wafer so that each surrounds and encloses the probe array of a biological array. FIG. **5** depicts a cross-section of this embodiment, showing the wafer **510** having a substrate **520** (preferably transparent to light) and a surface **530** to which is attached an array of probes **540**. A channel wall **550** covers a probe array on the wafer, thereby creating well spaces **560**. The wafer can be attached to the body by any attachment means known in the art, for example, gluing (e.g., by ultraviolet-curing epoxy or various sticking tapes), acoustic welding, sealing such as vacuum or suction sealing, or even by relying on the weight of the body on the wafer to resist the flow of fluids between test wells.

In another preferred embodiment, depicted in cross section in FIG. **6**, the plates include a body **610** having preformed wells **620**, usually flat-bottomed. Individual biological chips **630** are attached to the bottom of the wells so that the surface containing the array of probes **640** is exposed to the well space where the sample is to be placed.

In another embodiment, the biological chip plate has a wafer having a plurality of probe arrays and a material resistant to the flow of a liquid sample that surrounds each probe array. For example, in an embodiment useful for testing aqueous-based samples, the wafer can be scored with waxes, tapes or other hydrophobic materials in the spaces between the arrays, forming cells that act as test wells. The cells thus contain liquid applied to an array by resisting spillage over the barrier and into another cell. If the sample contains a non-aqueous solvent, such as an alcohol, the material is selected to be resistant to corrosion by the solvent.

The microplates of this invention have a plurality of test wells that can be arrayed in a variety of ways. In one embodiment, the plates have the general size and shape of standard-sized microtiter plates having 96 wells arranged in an 8×12 format. One advantage of this format is that instrumentation already exists for handling and reading assays on microtiter plates. Therefore, using such plates in biological chip assays does not involve extensive re-engineering of commercially available fluid handling devices. However, the plates can have other formats as well.

The material from which the body of the biological chip plate is made depends upon the use to which it is to be put. In particular, this invention contemplates a variety of polymers already used for microtiter plates including, for example, (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, polypropylene, polystyrene, polycarbonate, or combinations thereof. When the assay is to be performed by sending an excitation beam through the bottom of the plate collecting data through the bottom of the plate, the body of the plate and the substrate of the chip should be transparent to the wavelengths of light being used.

The arrangement of probe arrays in the wells of a microplate depends on the particular application contemplated. For example, for diagnostic uses involving performing the same test on many samples, every well can have the same array of probes. If several different tests are to be performed on each sample, each row of the plate can have the same array of probes and each column can contain a different

array. Samples from a single patient are introduced into the wells of a particular column. Samples from a different patient are introduced into the wells of a different column. In still another embodiment, multiple patient samples are introduced into a single well. If a well indicates a "positive" result for a particular characteristic, the samples from each patient are then rerun, each in a different well, to determine which patient sample gave a positive result.

D. Biological Chips

The biological chip plates used in the methods of this invention include biological chips. The array of probe sequences can be fabricated on the biological chip according to the pioneering techniques disclosed in U.S. Pat. No. 5,143,854, PCT WO 92/10092, PCT WO 90/15070, or U.S. application Ser. Nos. 08/249,188, 07/624,120, and 08/082,937, incorporated herein by reference for all purposes. The combination of photolithographic and fabrication techniques may, for example, enable each probe sequence ("feature") to occupy a very small area ("site" or "location") on the support. In some embodiments, this feature site may be as small as a few microns or even a single molecule. For example, a probe array of 0.25 mm^2 (about the size that would fit in a well of a typical 96-well microtiter plate) could have at least 10, 100, 1000, 10^4, 10^5 or 10^6 features. In an alternative embodiment, such synthesis is performed according to the mechanical techniques disclosed in U.S. Pat. No. 5,384,261, incorporated herein by reference.

Referring to FIG. **8**, in general, linker molecules, ¯O—X, are provided on a substrate. The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which the probes are located. The substrate and its surface preferably form a rigid support on which the sample can be formed. The substrate and its surface are also chosen to provide appropriate light-absorbing characteristics. For instance, the substrate may be functionalized glass, Si, Ge, GaAs, GaP, SiO_2, SiN_4, modified silicon, or any one of a wide variety of gels or polymers such as (poly)tetrafluoroethylene, (Poly)vinylidenedifluoride, polystyrene, polycarbonate, polypropylene, or combinations thereof. Other substrate materials will be readily apparent to those of skill in the art upon review of this disclosure. In a preferred embodiment the substrate is flat glass or silica.

Surfaces on the solid substrate usually, though not always, are composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials. In one embodiment, the surface will be optically transparent and will have surface Si—OH functionalities, such as those found on silica surfaces.

A terminal end of the linker molecules is provided with a reactive functional group protected with a photoremovable protective group, O—X. Using lithographic methods, the photoremovable protective group is exposed to light, hv, through a mask, M_1, that exposes a selected portion of the surface, and removed from the linker molecules in first selected regions. The substrate is then washed or otherwise contacted with a first monomer that reacts with exposed functional groups on the linker molecules (¯T—X). In the case of nucleic acids, the monomer can be a phosphoramidite activated nucleoside protected at the 5'-hydroxyl with a photolabile protecting group.

A second set of selected regions, thereafter, exposed to light through a mask, M_2, and photoremovable protective group on the linker molecule/protected amino acid or nucleotide is removed at the second set of regions. The substrate is then contacted with a second monomer containing a photoremovable protective group for reaction with exposed functional groups. This process is repeated to selectively apply monomers until polymers of a desired length and desired chemical sequence are obtained. Photolabile groups are then optionally removed and the sequence is, thereafter, optionally capped. Side chain protective groups, if present, are also removed.

The general process of synthesizing probes by removing protective groups by exposure to light, coupling monomer units to the exposed active sites, and capping unreacted sites is referred to herein as "light-directed probe synthesis." If the probe is an oligonucleotide, the process is referred to as "light-directed oligonucleotide synthesis" and so forth.

The probes can be made of any molecules whose synthesis involves sequential addition of units. This includes polymers composed of a series of attached units and molecules bearing a common skeleton to which various functional groups are added. Polymers useful as probes in this invention include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either α-, β-, or ω-amino acids, heteropolymers in which a known drug is covalently bound to any of the above, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this disclosure. Molecules bearing a common skeleton include benzodiazepines and other small molecules, such as described in U.S. Pat. No. 5,288,514, incorporated herein by reference.

Preferably, probes are arrayed on a chip in addressable rows and columns in which the dimensions of the chip conform to the dimension of the plate test well. Technologies already have been developed to read information from such arrays. The amount of information that can be stored on each plate of chips depends on the lithographic density which is used to synthesize the wafer. For example, if each feature size is about 100 microns on a side, each array can have about 10,000 probe addresses in a 1 cm^2 area. A plate having 96 wells would contain about 192,000 probes. However, if the arrays have a feature size of 20 microns on a side, each array can have close to 50,000 probes and the plate would have over 4,800,000 probes.

The selection of probes and their organization in an array depends upon the use to which the biological chip will be put. In one embodiment, the chips are used to sequence or re-sequence nucleic acid molecules, or compare their sequence to a referent molecule. Re-sequencing nucleic acid molecules involves determining whether a particular molecule has any deviations from the sequence of reference molecule. For example, in one embodiment, the plates are used to identify in a particular type of HIV in a set of patient samples. Tiling strategies for sequence checking of nucleic acids are described in U.S. patent application Ser. No. 08/284,064 (PCT/US94/12305), incorporated herein by reference.

In typical diagnostic applications, a solution containing one or more targets to be identified (i.e., samples from patients) contacts the probe array. The targets will bind or hybridize with complementary probe sequences. Accordingly, the probes will be selected to have sequences directed to (i.e., having at least some complementarity with) the target sequences to be detected, e.g., human or pathogen sequences. Generally, the targets are tagged with a detectable label. The detectable label can be, for example, a luminescent label, a light scattering label or a radioactive

label. Accordingly, locations at which targets hybridize with complimentary probes can be identified by locating the markers. Based on the locations where hybridization occurs, information regarding the target sequences can be extracted. The existence of a mutation may be determined by comparing the target sequence with the wild type.

In a preferred embodiment, the detectable label is a luminescent label. Useful luminescent labels include fluorescent labels, chemi-luminescent labels, bio-luminescent labels, and colorimetric labels, among others. Most preferably, the label is a fluorescent label such as fluorescein, rhodamine, cyanine and so forth. Fluorescent labels include, inter alia, the commercially available fluorescein phosphoramidites such as Fluoreprime (Pharmacia), Fluoredite (Millipore) and FAM (ABI). For example, the entire surface of the substrate is exposed to the activated fluorescent phosphoramidite, which reacts with all of the deprotected 5'-hydroxyl groups. Then the entire substrate is exposed to an alkaline solution (eg., 50% ethylenediamine in ethanol for 1–2 hours at room temperature). This is necessary to remove the protecting groups from the fluorescein tag.

To avoid self-quenching interactions between fluorophores on the surface of a biological chip, the fluorescent tag monomer should be diluted with a non-fluorescent analog of equivalent reactivity. For example, in the case of the fluorescein phosphoramidites noted above, a 1:20 dilution of the reagent with a non-fluorescent phosphoramidite such as the standard 5'-DMT-nucleoside phosphoramidites, has been found to be suitable. Correction for background non-specific binding of the fluorescent reagent and other such effects can be determined by routine testing.

Useful light scattering labels include large colloids, and especially the metal colloids such as those from gold, selenium and titanium oxide.

Radioactive labels include, for example, ^{32}P. This label can be detected by a phosphoimager. Detection of course, depends on the resolution of the imager. Phosophoimagers are available having resolution of 50 microns. Accordingly, this label is currently useful with chips having features of that size.

E. Uses

The methods of this invention will find particular use wherever high through-put of samples is required. In particular, this invention is useful in clinical settings and for sequencing large quantities of DNA, for example in connection with the Human Genome project.

The clinical setting requires performing the same test on many patient samples. The automated methods of this invention lend themselves to these uses when the test is one appropriately performed on a biological chip. For example, a DNA array can determine the particular strain of a pathogenic organism based on characteristic DNA sequences of the strain. The advanced techniques based on these assays now can be introduced into the clinic. Fluid samples from several patients are introduced into the test wells of a biological chip plate and the assays are performed concurrently.

In some embodiments, it may be desirable to perform multiple tests on multiple patient samples concurrently. According to such embodiments, rows (or columns) of the microtiter plate will contain probe arrays for diagnosis of a particular disease or trait. For example, one row might contain probe arrays designed for a particular cancer, while other rows contain probe arrays for another cancer. Patient samples are then introduced into respective columns (or rows) of the microtiter plate. For example, one column may be used to introduce samples from patient "one," another column for patient "two" etc. Accordingly, multiple diagnostic tests may be performed on multiple patients in parallel. In still further embodiments, multiple patient samples are introduced into a single well. In a particular well indicator the presence of a genetic disease or other characteristic, each patient sample is then individually processed to identify which patient exhibits that disease or trait. For relatively rarely occurring characteristics, further order-of-magnitude efficiency may be obtained according to this embodiment.

Particular assays that will find use in automation include those designed specifically to detect or identify particular variants of a pathogenic organism, such as HIV. Assays to detect or identify a human or animal gene are also contemplated. In one embodiment, the assay is the detection of a human gene variant that indicates existence of or predisposition to a genetic disease, either from acquired or inherited mutations in an individual DNA. These include genetic diseases such as cystic fibrosis, diabetes, and muscular dystrophy, as well as diseases such as cancer (the P53 gene is relevant to some cancers), as disclosed in U.S. patent application Ser. No. 08/143,312, already incorporated by reference.

The present invention provides a substantially novel method for performing assays on biological arrays. While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those of skill in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

What is claimed is:

1. A method for making a biological chip plate comprising the steps of:

(a) providing a body comprising a plurality of wells defining spaces;

(b) providing a wafer comprising on its surface a plurality of probe arrays, each probe array comprising a collection of probes, at least two of which are different, arranged in a spacially defined and physically addressable manner;

(c) attaching the wafer to the body so that the probe arrays are exposed to the spaces of the wells.

2. The method of claim 1 wherein the probes are DNA or RNA molecules.

3. A method for making a biological chip plate comprising the steps of providing a wafer comprising on its surface a plurality of probe arrays, each probe array comprising a collection of probes, at least two of which are different, arranged in a spacially defined and physically addressable manner; and applying a material resistant to the flow of a liquid sample so as to surround the probe arrays, thereby creating test wells.

4. The method of claim 3 wherein the probes are DNA or RNA molecules.

* * * * *